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THE NORMAN ASHTON

Proctor Medal Award

PROCEEDINGS

of the

Association for Research in Ophthalmology, Inc.

Twenty-sixth Meeting

New York, New York

June 3, 4, 5, and 6, 1957

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NORMAN ASHTON, M.R.C.P., M.R.C.S.

REMARKS ON ACCEPTANCE OF THE PROCTOR MEDAL AWARD

June, 1957

NORMAN ASHTON, M.R.C.P., M.R.C.S.
London, England

Of all the honors to be gained in ophthalmologic research in this country, the Proctor Award is the most highly prized, and justly so, for its recipient is selected from a group of workers that can boast increasingly fine achievement, and the names of those elevated to this aristocracy through the years have added much luster to the medal. Who then would not feel overwhelmed on receiving such an honor? Certainly I do. But it would be foolish to deny my pleasure in this good fortune or my pride in being the first non-American to receive the reward, and I thank you with all my heart for your kindness.

As a pathologist in a world of ophthalmologists, I have been generously and warmly welcomed, so it is with some shame that I recall the misgivings I had on leaving general pathology eight years ago. In my ignorance it seemed that the eye was quite inadequate a structure to provide a life's work in pathology, especially since the subject had apparently been thoroughly explored for a century or so by clinicians in their spare time. Nor may I say were these misgivings entirely on my side! But in pathology the emphasis on descriptive morbid anatomy ("collecting butterflies" as Professor Cameron calls it) is diminishing and the modern pathologist is no longer content to speak of gross appearances, such as "sago spleen" or "nutmeg liver," but searches rather for the explanation of abnormal function, and in so doing has moved into a cellular cosmos where the cell itself must be dissected. To all the old techniques of investigation have been added a myriad of new ones, and, in exploiting them, a single cell—or even one of its organelles—could provide a life-time study. How rich then is the eye in opportunity, containing as it does so many types of cell and so many specialized structures of its own, arranged with such nicety that pathologic changes may be seen, both in vivo and in vitro, with greater clarity than in almost any other tissue. So rich is this field that there is a danger of dissipating one's effort down a dozen attractive pathways and for myself I find the greatest difficulty in selecting the particular problems upon which to concentrate.

Indeed there is so much work to be done that it would be better if as a profession we spent less time writing in journals and talking at meetings, but on a special occasion like this perhaps I, a particular offender, may be forgiven for luxuriating in the space you have provided by contemplating the future of the subject in which I have been so signally honored.

Ocular pathology has always been an esoteric learning, pigeonholed as it were in the orbit and held sacrosanct by the foreign language of its terminology, so that to become versed in it is as much a linguistic as a pathologic exercise. It is a branch of knowledge created by ophthalmologists in the unhurried days before the First World War, and, like their contemporaries in general pathology, their contributions were mainly descriptive in character. And what excellent contributions they were! The perfection of the classical papers and the beauty of the illustrations are still a matter of wonder and admiration. Those halcyon days, however, are over and it is debatable whether the practice of ocular pathology should continue solely as a part-time occupation of persons trained primarily as clinicians.

If in the future, eye pathology is to be taught and practiced in the traditional way, as an elaborate recording of histologic minutia, then the subject is not too demanding and may well be undertaken as a part-time

pursuit, and probably best by the ophthalmologist who is most able to extract the full clinical value from the findings. But if the study of ocular pathology is to have its full meaning, the eye must be regarded as a unit of an entire organism, and its behavior in disease must as far as possible be related to that of the whole. Research in this field, in common with the general tendency, should concern itself with disease mechanisms rather than with disease patterns, and for this purpose the widest possible knowledge of pathologic processes is desirable and the whole armamentarium of modern scientific method should be available. To establish ocular pathology on this broad basis will demand the full and concentrated attention of workers trained and experienced in the appropriate disciplines.

By now you will be suspecting that I am about to claim that the most suitable persons to be in charge of this work are full-time pathologists. So I am. But it would be most forgetful of the help I have personally received from ophthalmologists, and indeed most insensitive on an occasion such as this, to suggest that the future of ocular pathology lies solely with the pathologist. Clearly the department of ocular pathology would suffer considerably if deprived of clinical experience, but it seems to me that there are at least three ways in which this could still be available.

First, through the provision of facilities for senior clinicians to work in the laboratory on problems in which they are particularly interested; secondly through part-time or full-time posts for younger clinicians working on a thesis or collaborating in research, and thirdly through contact with postgraduate students or visitors attending the laboratory for study. In short, ocular pathology is best carried out by close co-operation between the clinician and the laboratory worker, but the emphasis I think should be upon pathology and not upon the eye.

The work from my own department which you have so kindly thought worthy of recognition derives entirely from this system. On the pathologic side there are, or have been until recently, Dr. Hugh Greer (now director of pathology at the Melbourne Eye and Ear Hospital), Dr. Charles Smith (now head of a trachoma unit), Dr. Patrick Schofield, Dr. William Henderson, and Dr. Christopher Pedler. On the ophthalmologic side there are Mr. Charles Cook, Mr. Kenneth Wybar, Mr. Redmond Smith, Mr. Trevor-Roper, and Mr. Geoffrey Serpell and Mr. Basil Ward (now in Australia). To all these excellent enthusiasts, each outstanding in his own way, I am deeply indebted, and were it not lèse majesté to deface the Proctor Medal it should in justice be divided among us.

Professor Pulvertaft, who taught me pathology as a student, gave me this advice when I set off for the Institute of Ophthalmology in 1948. "Never talk dud ophthalmology to pathologists or dud pathology to ophthalmologists." This pithy counsel has been my watchword, nor do I believe that with both specialists on my doorstep, would I have the courage to deviate from it.

I shall carry the medal home to London as a trophy to share with my colleagues at the Institute, and particularly with those of my own department who have so greatly contributed to the work cited for the award. In conclusion I would especially like to acknowledge my indebtedness to Sir Stewart Duke-Elder, director of the Institute, who has always encouraged and helped me in every way, to the late Mr. Eugene Wolff who taught me the elements of ocular pathology, to Mr. Charles Cook who has worked with me from the beginning, and lastly to Mr. George Knight, the chief technician, and Miss FitzGerald, the secretary of my department, whose loyalty and selfless devotion to their work have been of inestimable value to me personally.

RETINAL VASCULARIZATION IN HEALTH AND DISEASE

PROCTOR AWARD LECTURE OF THE ASSOCIATION FOR RESEARCH IN OPHTHALMOLOGY

NORMAN ASHTON
London, England

It was my intention to speak today on the whole problem of new vessel formation in the eye, but owing to several new observations made in my department just before I left, which I would like to report to you, it will be necessary to confine my remarks to vessel growth in the retina.

The nature of the stimuli which excite and direct the growth of blood vessels into the retina, in both normal and abnormal circumstances, is as yet unknown, but as a direct result of laboratory studies of retrolental fibroplasia we have been inundated with a mass of new and apparently isolated facts. It is imperative to arrange this knowledge in some sort of order, and a few broad generalizations appear to be justified on the data at present available, but they should not be regarded as rigid conclusions until the facts are incontrovertible, and that cannot at present be claimed. I present them to show the direction in which our experimental results are leading us and to provide working hypotheses to knit scattered observations together as a guide, and possibly a stimulus, to future research.

NORMAL RETINAL VASCULARIZATION

The mode of development of the human retinal circulation has been known for many years and need not be described here, but there is one feature which until recently has not been recorded. It has always been taught that the retinal vessels bud out as fully formed capillaries from the central retinal artery at the disc, where it is continuous with the hyaloid artery during the fourth month of intrauterine life; one bud appears from the upper side and one from the lower side, which then grow out into the nerve-fiber layer of the retina (fig. 1).

The feature which has only recently been appreciated, however, is that these vascular buds are preceded and accompanied by clusters of undifferentiated cells (Ashton, 1954[a]), presumably mesenchymal elements derived from the vessel wall. They may readily be identified with PAS stain as they contain PAS-positive granules—identified as glycogen (Serpell, 1954)—which are absent in endothelial cells. They always lie at the periphery of the advancing vessels as a spearhead of the ingrowth.

Whether these mesenchymal cells are responsible for the formation of the whole retinal vasculature, or only those elements, such as muscle and connective tissue which cannot derive from endothelium is not known with certainty. It is interesting that in retrolental fibroplasia wherein the blood vessels proliferate abnormally, these cells are similarly affected, and may play an important role in the subsequent formation of fibrous bands in the vitreous.

Fig. 1 (Ashton). Injected retina from a 20-week fetus viewed from one side. Above and below the hyaloid artery, vessels may be seen budding into the retina. (×20.)

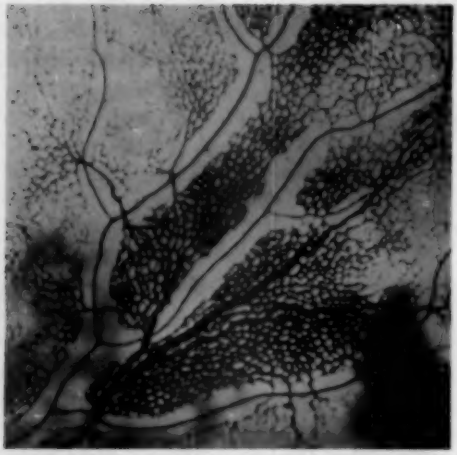

Fig. 2 (Ashton). Injected retina from a one-day-old kitten, showing capillary development from the veins, which occurs predominantly from the side of the vein remote from the artery. There is a capillary-free zone around the arteries. (×33.)

The comparatively recent studies of Michaelson (1948 a and b), particularly in man, the rat, and cat, by providing new observations and by emphasizing old ones have been of great value in indicating the general principles involved in normal retinal vascularization. He showed that the formation of retinal capillaries is pre-eminently a function of the retinal veins and that if a vein and artery are close to each other, growth takes place predominantly from the side of the vein remote from the neighboring artery (fig. 2). He pointed out the capillary-free zone existing around the arteries, both in the immature and in the mature retina (fig. 3), a feature previously depicted by Leber (1903).

Fig. 3 (Ashton). Adult human retina injected with India ink to show the capillary-free zone around the artery. (×105.)

To Michaelson these anatomic facts were clearly associated with each other and suggested to him the presence in the retina of a vasoformative factor, possibly biochemical in nature, which was present in gradients of concentration differing in arterial and venous neighborhoods. He did not speculate further upon the nature of this factor, but quite clearly the pattern of vascular growth very strongly suggests its possible association with the level of oxygen tension within the tissues.

Indeed, the only adequate explanation of all the observed features of vessel growth and architecture in the normal retina is that a vasoformative factor—simple or complex—arises in areas of lower oxygen tension, but not in areas of higher oxygen tension. Thus it becomes apparent why vessels invade the developing retina at the 100-mm. stage as the choroid fails to meet its increased oxygen requirements; why growth is predominantly on the venous side; why there is a peri-arterial capillary-free zone; and why vessels never transgress, despite the absence of an anatomic barrier, the macula, the outer layers, or the periphery of the retina which are adequately oxygenated from the choriocapillaris.

These conclusions, however, are no more than speculative deductions from anatomic observations and I shall now consider experimental work which more fully explores the hypothesis.

Experimental studies

The idea that vascular morphogenesis in the retina may be related to oxygen was first expressed in the literature by Campbell

(1951), who tested the hypothesis by placing one-day-old rats in a low-pressure environment, and made the important observation that the capillary-free zone around the arteries became significantly narrower than in control animals. The converse of this finding, that raised oxygen tensions lead to widening of the capillary-free zone, we demonstrated (fig. 4) later when investigating the effect of hyperoxia on the retina of the kitten (Ashton et al., 1954).

There would thus appear to be two clearly defined processes in retinal vascular growth; one of inhibition, as evidenced by *vaso-obliteration* occurring in high oxygen concentrations, and one of stimulation, as evidenced by *vasoproliferation* occurring in low oxygen concentrations. The two processes, however, are not perfect mirror images of each other, for whereas in vasoproliferation both the normal and abnormal forms appear to be due to a similar mechanism, vaso-obliteration cannot be regarded simply as an extension of the normal periarterial capillary-free zone, for this is a region into which vessels have never grown and cannot, therefore, have been obliterated. Thus vaso-obliteration probably does not exist as a normal mechanism.

Since the data relating to these two processes are extremely intricate I shall consider them separately.

1. Vaso-obliteration

That oxygen in high concentrations can produce a partial or total obliteration of the retinal vessels in the immature eye was discovered about four years ago in experiments on the kitten (Ashton et al., 1953) and much has been learned about the phenomenon since that time. It commences as a marked constriction of the arteries and arterioles; the arterioles and arterial side of the capillary circulation are the first vessels to obliterate; this is followed by disappearance of the whole capillary bed; then the main arteries and finally the main veins close, leaving an apparently avascular retina.

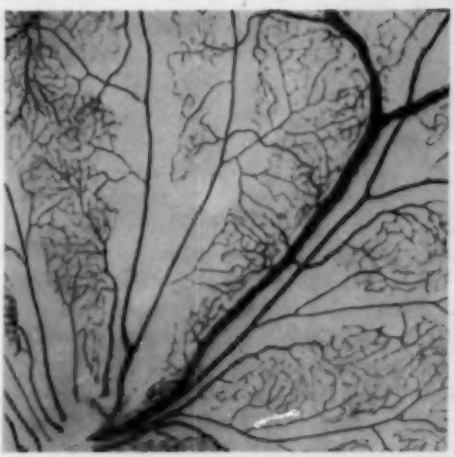

Fig. 4 (Ashton). Injected retina from an 18-day-old kitten subjected to hyperoxia. Vaso-obliteration is in its early stages and is seen to begin as a widening of the peri-arterial capillary-free zone. (×33.)

Injected specimens show that in general this process begins peripherally and spreads posteriorly, the disc region being the most resistant and the last area to be completely obliterated. It should be realized, however, that injected specimens do not reveal the degree of closure of the vessels in life, but only their ability to re-open when injected. This distinction is important, because it means that vaso-obliteration can only be studied accurately by direct observation in the living animal.

This phenomenon is confined to the growing retinal vessels, occurring neither in the other vessels of the eye nor in those of other organs (Ashton et al., 1954; Patz, 1954); nor in growing vessels as observed in the rabbit cornea (Ashton and Cook, 1954; Michaelson et al., 1954), nor in the rabbit's ear-chamber (Ashton and Cook, 1954); nor in chick embryos; nor in cat or rat embryos (Ashton, 1954b). Nor indeed does it occur in the retina itself, when it becomes detached from the choroid.

As shown by injections, the severity of the vaso-obliterative effect of oxygen is inversely proportional to the maturity of the

TABLE 1

SUMMARY OF EXPERIMENT

Group	Age (days)	Oxygen Exposure (days)	Air (days)	Vasoproliferation
A.	1	4	10	In 15-day-old retina
B.	10	15	10	In 35-day-old retina

retinal vessels and, once the circulation is fully established and the normal vascular architecture attained, oxygen fails to produce significant obliterative changes. Oxygen thus has no structural effect on the vessels of the adult cat, and recent experiments suggest that this is due, at least in part, to a change in the retinal metabolism.

It was possible, for instance, by exposing kittens of differing ages to varying periods of oxygen, to obtain, on transfer to air, vasoproliferations in retinas of an age normally sensitive to oxygen, and vasoproliferations in retinas of an age normally insensitive to oxygen (table 1). Re-oxygenation showed that obliteration of these abnormal proliferations occurred only in the first group. This demonstrates in a rather striking way that vaso-obliteration may be the outcome of an interaction between oxygen and retinal metabolism and not a peculiar reaction of growing retinal vessels themselves (Ashton, 1956).

Vaso-obliteration occurs in two phases, *immediate* and *delayed*. An *immediate* effect, consisting of severe constriction of the large vessels with obliteration of the capillaries, develops after five minutes' exposure to hyperoxia. The vessels then dilate again after about 10 minutes and so remain, although in continuous oxygen, for about six hours when the *delayed* effect comes into operation. This results in a gradual total vaso-obliteration and once it is achieved the circulation may be either opened or closed, in each case in about five minutes, by alternating air and oxygen. But after longer periods the process gradually becomes irreversible and at the end of three days the vessels seem to have largely disappeared although their remnants can be made out in flat preparations and in sections. The time relationships of these changes suggest that oxygen is inhibiting or poisoning an enzyme system vitally concerned in the maintenance of the vessels' patency.

In considering the possible mechanisms involved one could first postulate the elaboration of a vasoconstrictor substance. It would, however, be difficult to imagine upon what cells in such a primitive vascular system it could act, especially since known vasoconstrictors do not obliterate growing retinal vessels, nor is sympathetic innervation concerned in the mechanism (Patz, 1955; Cook and Ashton, 1955). Furthermore, extracts of retinas in which vaso-obliteration had been induced (extracts prepared in high ambient concentrations of oxygen) have been found to have no effect when injected via the vitreous onto normal retinas in direct-observation experiments on the living animal (Ashton and Pedler, 1957). Nor did such extracts show any evidence of a smooth muscle stimulant as tested against preparations of rat colon in two experiments (Dr. N. Ambache kindly carried out these tests on the extract with which he was provided).

Secondly one could postulate the *removal of a vasodilator substance* inherent in the vasoformative process. It is noteworthy, however, that for growing vessels to be obliterated by hyperoxia it is essential for them to be actually within the retinal tissue; developing vessels within the vitreous, on the iris, or in the cornea are not affected in this way. If oxygen produced vaso-obliteration merely by neutralizing a vasoformative substance it is hard to understand why it should be so remarkably selec-

tive. It is a difficult question to investigate and we have no conclusive evidence either to deny or to support the possibility.

Thirdly, one must consider that the vessels may close through *external pressure*. An increase of intraocular pressure as a result of hyperoxia has been excluded, but could the process of vaso-obliteration be partly or wholly due to swelling of the cells surrounding the blood stream?

Although it is known that endothelial cells have the property of swelling when subjected to irritating stimuli (Krogh, 1929), we can probably exonerate them here as oxygen vaso-obliteration does not occur in growing vessels outside the retina. Swelling of the retinal tissue, however, remains a possibility, and, in considering one of the ways in which this might theoretically occur, we were led to some new and interesting findings. The hypothesis upon which our experiments were based was as follows.

Until recently it has been the orthodox view that the cells of the body are in osmotic equilibrium with their extracellular environment, that is, their contents are isotonic with the blood plasma. It would now appear that this is the exception rather than the rule (Bartley et al., 1954); in fact it has been estimated that the osmotic pressure of the cell fluids is normally 50 to 100 percent greater than that of the extracellular fluid (Robinson, 1950). If these findings are substantiated, the idea of the osmotic equilibrium being governed solely by the physical properties of cell membranes would no longer be tenable, and it would be necessary to postulate that the water content of the cell, and therefore the cell size, is dependent upon some dynamic process involving a continuous supply of energy.

That this concept of osmoregulation may be correct is suggested, for instance, by the ability of some cells of the renal tubules to maintain a normal internal environment despite the variable tonicity of the urine which bathes them. In fact, this consideration led Robinson (1950) to investigate the behaviour of tissue slices cut from the kidneys of normal adult rats. By measuring the oxygen consumption and the amount of water in the tissues under varying conditions, he found that respiration was more important than the osmotic pressure of the external media in determining the amount of water in the cells. When respiration was inhibited by cyanide, water passed into the cells. In short, the energy for the transport of water across the cell membrane was in this case derived from cell respiration. It is interesting to note in his experiments that the imbibition of water was a rapid process, being almost complete in two minutes and then maintained for several hours.

It has been suggested that this steady-state exchange depends on energy-driven "pumps" located in the mitochondria, as these structures when isolated from the cell can be shown to do osmotic work by moving substances against concentration gradients (Bartley et al., 1954). Although there is not complete agreement on the existence of these pumping mechanisms, it having been denied that the cell is significantly hypertonic, and claimed that cellular swelling is isosmotic in nature (Mudge, 1956), the cardinal point that tissue cells swell when placed in an environment where the metabolism is inhibited is not in question.

Some years ago Trowell (1946) showed that liver cells when subjected to anoxia increased in size due to the passage of water into the cells. This process he described as "intracellular edema," although in this instance he did not attribute it to osmotic absorption.

Returning now to the retina, it will be recalled that it has the highest rate of respiration of any tissue (Warburg, 1927) and a higher rate of glycolysis in air than most other tissues. As early as 1924 it had been suggested by Warburg et al., that the various layers of the retina possessed their own peculiar metabolism, some of the cells being responsible for the high glycolytic activity, while the others were almost completely

oxidative in character. This suggestion was later strongly supported by other workers (Sjöstrand, 1953; Strominger and Lowry, 1955; Lowry et al., 1956).

In both monkey and rabbit, it would appear that the rod and cone layer is predominantly concerned with oxidative metabolism while the inner layers, especially of avascular retinas, as in the rabbit, are predominantly glycolytic in activity. Now the immature retina, at a stage when oxygen vaso-obliteration may be obtained, differs from the mature retina in the important respect that retinal vascularization is still developing, so that it is reasonable to suppose that the metabolism of the inner layers of such a retina may be predominantly glycolytic. Indeed, we have already deduced from our experiments that the immature retina probably has a metabolism differing from that in the mature retina.

If such is the case it would follow that fluid exchanges through the walls of the inner retinal cells—and, therefore, the cell size—might be dependent upon energy derived, not from respiration, but from glycolysis. With these considerations in mind we argued that if swelling of the cells is an important factor in the obliteration of retinal vessels by oxygen, then the inhibition of glycolysis by known enzyme poisons might also lead to vaso-obliteration.

We are, therefore, investigating the action of sodium fluoride which is known to arrest glycolysis by inhibiting enolase through displacing magnesium from the enzyme, and it has been shown that this inhibition occurs particularly in retinal tissue (Dickens and Greville, 1932; Holmes, 1940; Kerly and Bourne, 1940; Lenti, 1940).

By directly observing the developing vessels in the retina of the kitten through a limbal window, we found that sodium fluoride in isotonic solution, when injected into the vitreous, produced vaso-obliteration in a few minutes and in the same order of vessel closure as that seen on exposure to hyperoxia. As in the case of oxygen, this effect is apparently confined to the vessels of the immature retina and experiments on the adult retina and on extraocular vessels have so far been negative.

The first change noted after introducing the fluoride was a remarkable engorgement of the veins, which, as in the oxygen effect, were the last to close. These observations appear inconsistent with a closure from external compression, for one would expect vessels with the lower pressure to close first. It was most interesting to find, however, that their engorgement is associated with a compression of the veins in the disc region which could well be due to an increased tension within the retina from intracellular edema (fig. 5). The vessels rapidly reopened when hypertonic solutions of salt or sucrose were injected into the vitreous, which supports the notion that vessel closure may be due to swelling of the cellular environment. An exactly similar result may be obtained with isotonic solutions of iodoacetic acid.

These experiments are still in their very early stages and are being reported in detail elsewhere (Ashton et al., 1957) but it is of interest to know that oxygen vaso-obliteration can be so closely mimicked by other drugs and it is tempting to believe that the two processes share a common mechanism. Although there is little evidence that this is so, I think we have some indication that oxygen vaso-obliteration, as seen in the immature retina, may be due, not to active vasoconstriction or to removal of a vasodilator, but to a passive closure from swelling of the retinal environment; a swelling which theoretically could result from the inhibition by oxygen of some enzyme system upon which glycolysis depends.

As far as I am aware, this concept is a new one, but I introduce it with the important reservation that at present we have practically no direct evidence that swelling actually occurs in the retinal tissue after fluoride or oxygen exposure. Nor have we yet shown that the hypothetical considera-

Fig. 5 (Ashton). Retina showing the fluoride effect on the retinal vessels in the disc region. The disc and hyaloid artery may be seen on the extreme left of the picture. Note that the optic nerve fibers stand out distinctly, the arteries and capillaries are largely obliterated, while the vein is markedly engorged and constricted as it enters the disc. (×94.)

tions which led us to devise these experiments, namely the inhibition of glycolysis with a breakdown in osmoregulation, are in fact those operating in the fluoride effect. However, if future work substantiates this concept, it will not only have considerably clarified the enigma of the action of oxygen on the immature retina, but it will have uncovered a pathologic process which I feel sure would be important in interpreting disease pictures in the retina.

2. Vasoproliferation

It will be recalled that when a kitten is returned to air after several days in high oxygen, the obliterated vessels are unable to re-open to any satisfactory extent, and new vessels then invade the retina and extend into the vitreous. Similar proliferations have been obtained by other workers in newborn mice, ratlings, kittens, and puppies (Patz et al., 1953; Patz, 1954; Gyllensten and Hellström, 1954). These workers, and others interested in the problem of retrolental fibroplasia, have sought to explain these vascular proliferations in terms of oxygen poisoning leading to inactivation of oxidative enzymes, particularly of succinic dehydrogenase; that is, they have postulated that the proliferations are caused by an anoxia which is histotoxic in type.

On the other hand, I have always believed that the vasoproliferative reaction results purely from obliteration of the vessels and is not in itself directly concerned with oxygen exposure. This distinction is

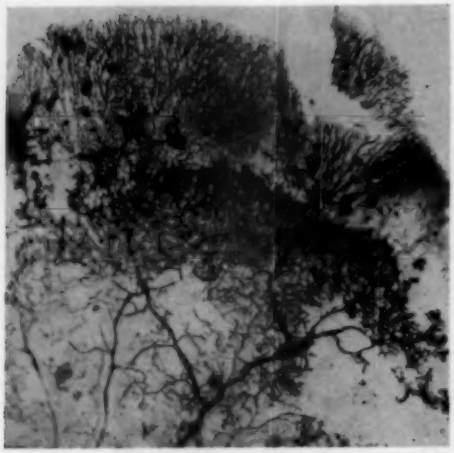

Fig. 6 (Ashton). Injected retina showing the type of vasoproliferation obtained in a more mature kitten when oxygen vaso-obliteration is only peripheral. The subsequent growth of vessels is similarly located. (×23.)

of some importance in understanding the general phenomenon of vasoproliferation in the eye, and, although the evidence is not yet conclusive one way or the other, my contention is supported by a number of facts.

First, as shown by our direct-observation experiments, the effect of oxygen is rapidly reversible at a stage when the vessels are still able to re-open; it becomes irreversible only when closure of the vessels has been maintained for so long that the vessel walls have become adherent or have been absorbed. In other words the action of oxygen appears to be only inhibitory and confined to the actual period of exposure. On this evidence it would appear doubtful whether oxygen at atmospheric pressure can give rise to enzyme-poisoning which persists on transfer to air. It will be recalled that in our experiments where kittens were allowed to survive for a year or two after total oxygen vaso-obliteration with reformation of the whole retinal vasculature, there was apparently no permanent impairment of vision although the vascular pattern was abnormal.

Secondly, vasoproliferation is always proportionate to the degree of vaso-obliteration and arises from those vessels immediately adjacent to the obliterated areas. Thus, in the youngest animals where obliteration is total, vasoproliferation recommences from the disc; in semimature animals where closure is partial, vessel growth occurs from the unobliterated vessels in the posterior fundus; and in more mature animals where obliteration is only peripheral, the subsequent vasoproliferation is similarly confined (fig. 6).

Thirdly, when irreversible vaso-obliteration is prevented, vasoproliferation does not occur, although the animal be exposed to the same concentration and period of hyperoxia as in the standard experiment. For instance, permanent closure of vessels can be prevented by administering anticoagulants which prevent the vessel walls adhering (not, incidentally, a very reliable method), or by re-opening the vessels periodically by giving the oxygen intermittently, that is by returning the animal to air for one hour after every period of five hours' exposure to oxygen. In both cases transfer to air does not produce vasoproliferation. Hence an essential factor determining the abnormal growth of vessels appears to be obliteration of the retinal vessels, and since they do not proliferate in oxygen but only on transfer to air, it follows that hypoxic conditions are also required.

Taking all these facts into consideration it is reasonable to conclude that the factor which stimulates the growth of vessels in the retina is formed in hypoxic tissue, and it is logical to speculate that it probably derives from the products of anaerobic metabolism. It is as though the mechanism of vessel-formation were especially designed to ensure the drainage of these products, and at the same time to replace the relatively wasteful process of anaerobic glycolysis by the more productive one of aerobic oxidation.

It might be expected that conditions of generalized anoxia, as result from low oxygen-tensions in the blood, from anemia or cardiac failure, could directly initiate retinal neovascularization. This is the mechanism

which has been advanced to explain those cases of retrolental fibroplasia which had not received supplemental oxygen. There is, however, little convincing experimental evidence to support this view. Kittens, ratlings, and newborn mice have been exposed to low ambient concentrations of oxygen for long periods (Ashton et al., 1954; Patz, 1955; Gyllensten and Hellström, 1955) but abnormal retinal vasoproliferations have not been produced. The important point here, I believe, is that although the necessary degree of hypoxia may be produced in the tissues, the efficiency of the circulation itself is not impaired, so that the vasoformative factor rapidly drains away and fails to accumulate to an active level.

From studies on the immature retina there are thus three basic requirements for the stimulation of vessel growth, and each of these is essential:

1. The presence of living cells to ensure an active metabolism.

2. A low oxygen tension to promote anaerobic metabolism.

3. A poor venous drainage to permit the accumulation of anaerobic metabolites, wherein the vasoformative factor probably lies.

In the mature retina, however, it is not easy to arrive at such clear-cut conclusions, because as already deduced the metabolic processes apparently differ. In fact, judging by the rapidity with which blindness follows occlusion of the central retinal artery the inner layers of the retina at least must have become largely dependent upon aerobic metabolism. Theoretically it would therefore follow that the vasoformative factor should be less readily elaborated in the mature than in the immature retina. That this is true, at least in certain circumstances, can be demonstrated simply by detaching the retina in experimental animals.

In the immature retina this procedure results in a profuse proliferation of vessels both outward to form a plexus of vessels on the under surface of the retina and inward to form vascular tufts in the vitreous.

In the mature animal detachment is not followed by new-vessel formation. It is, therefore, clear that the vasoformative stimulus is much less readily evoked in the adult animal.

Vasoproliferation in ocular disease

Nevertheless, as is well known, new-vessel proliferations are common features of several retinal diseases, so that the facility for elaborating the stimulus cannot be entirely lost, and it may be shown that the same basic requirements are as essential for vascular growth in the mature as in the immature retina. In contrast to the immature retina, however, *intraretinal* neovascularization in the mature retina is a rarity, although new-channel formation through obliteration and dilatation of pre-existing capillaries is common and has been misinterpreted as neovascularization. The vast majority of new-vessel formations arising from the adult retina extend almost immediately into the vitreous, either as delicate fronds of new vessels or in association with dense fibrous tissue.

In human eye disease, Klien (1938) based her classification of retinitis proliferans upon these two types. In Type I, exudations or hemorrhages into the vitreous of inflammatory or traumatic origin are followed by connective tissue proliferation and lastly by vascular ingrowth. Here it may be suggested that the vasoformative factor arises from the living cells carried into the vitreous, namely fibroblasts, which proliferating in an avascular tissue give rise to the necessary accumulation of anaerobic metabolites. Indeed, it is clear from histologic studies that the vascular component in this type of retinitis proliferans is proportional to the cellular content, and where the exudate is predominantly fibrinous there may be only a few tenuous vessels and sometimes even none.

In Klien's Type II retinitis proliferans there is a primary formation of new vessels with a secondary production of delicate con-

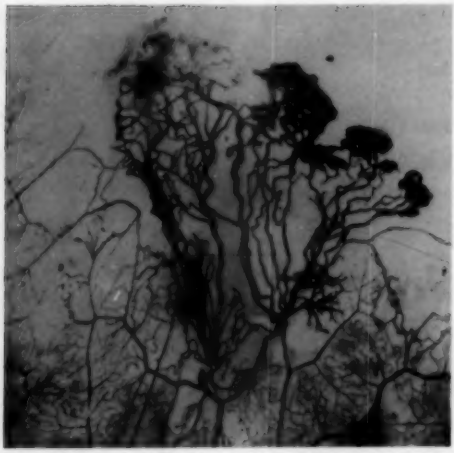

Fig. 7 (Ashton). Injected human retina from a case of Eales's disease showing that the intravitreal vasoproliferations are associated with vaso-obliteration in the underlying retina. (×13.)

nective tissue. This is the type of proliferating retinitis seen typically in diabetes and occlusion of the central retinal vein, although Type I may also occur. In pathologic specimens I have found that these intravitreal proliferations are always associated with underlying areas in the retina of obliterated capillaries or obstructed veins (fig. 7). Here it may be suggested that the vasoformative factor is elaborated in the hypoxic retinal tissue, and, failing to drain away, seeps into the vitreous where it reaches a concentration sufficient to stimulate the vessels to grow inward. They are exactly comparable to the glomerular tufts seen in experimental and natural retrolental fibroplasia and, similarly, may disappear when the stimulus has been expended or removed.

The concept of a preformed factor diffusing through the vitreous is applicable also to the problem of neovascularization of the iris. It is certainly not without significance that new vessels on the iris so frequently follow the development of retinal disease, wherein the pattern of vascular involvement provides the conditions for production of the vasoformative factor. Examples are: diabetic retinopathy, venous occlusion, Eales's disease, retinal detachment, and retrolental fibroplasia. In all these diseases the vasoformative factor may be elaborated either in the hypoxic retina or by fibroblasts in the vitreous, and be carried forward to stimulate the growth of vessels anteriorly.

In central venous occlusion, Redmond Smith (1954) has made the interesting observation that neovascularization at the disc tends to prevent vascularization at the filtration angles, which suggests that if the vasoformative factor is drained away by the intravitreal vessels, it cannot accumulate in sufficient quantity to reach the vessels in the anterior chamber.

These arguments could be even further elaborated, but sufficient examples have been quoted to justify the hope that the time may not be far distant when the mechanism of vaso-obliteration will be understood and the nature of the vasoformative factor identified. It is no exaggeration to prophesy that this knowledge should certainly provide new approaches in interpretation, and possibly new lines in treatment, of vascular diseases of the eye.

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BIOGRAPHICAL DATA

Norman Ashton, M.R.C.P., M.R.C.S.

Norman Ashton was born in London, England, on September 11, 1913. His adolescence was spent in the search for an outlet for his quite unusually varied talents. Eventually—and fortunately for ophthalmology—in 1933 he entered King's College, London, as a medical student and received his clinical training in the Westminster Hospital. As a medical student he is chiefly remembered for his artistic tendencies; and today the walls of the school are hung with a series of cartoons of his teachers of such merit and so delightfully scurrilous that their preservation was deemed necessary; today his paintings in oils are seen on the walls of his unique house in the 13th century Cloisters of Westminster Abbey as well as in the Institute of Ophthalmology, and are regularly exhibited in the art galleries of London. Moreover, during his graduate days he wrote—and the writing included the lyrics and the music—the annual pantomime that formed the social culmination of each academic year. Incidentally, or so it seemed, he graduated in medicine; and thereafter, apparently being found indispensible to the life of the hospital and its medical community, he completed an elaborate graduate training, acting for successive periods as an intern in the casualty department, in surgery and medicine, as well as in the departments of pediatrics, dermatology, and venereology, to become eventually the Senior Resident Medical Officer of the hospital, and finally, in 1939, a pathologist.

The Second World War then started and, in 1941, when England was expecting invasion, he went to that dangerous corner, the southeast coast of Kent, as blood-transfusion officer and pathologist; but as the

event turned out the only post-mortem material was provided by the local English (he did some 1,500 of them and escaped the consequences of five professional murders), and so he joined the Army. Posted initially to West Africa, he was shortly transferred to the Middle East where as a Lieut. Colonel, he occupied the position of officer commanding the Central Pathological Laboratories in this important theater. Here, in addition to the pathology of a general military hospital of over 1,000 beds, he was responsible for the pathologic services over a wide area, acted as referee in difficult and obscure cases, and undertook the training of selected medical officers and technicians in this specialty: as a rule he was never dealing with less than 60 trainees at any one time. Here also he carried out his first major research—on the problem of typhoid carriers in locally employed Egyptian food-handlers; the results of this work were important and have been incorporated into the permanent practice of the army.

Demobilized from the army in 1948, he rejoined his own hospital in England, but some months thereafter an event of some significance to ophthalmology occurred. At that time the writer was acting as obstetrician at the birth of the Institute of Ophthalmology in London, and it seemed to him that the post of pathologist was one of the most crucial. He was worried about the general state of ophthalmic pathology, the steady progress of which seemed to have been retarded by the fact that it had always been served by young ophthalmologists as a temporary expedient while waiting for consulting appointments or as a side-line thereafter. He was looking for someone with a wide knowledge of general pathology, someone who was steeped in its philosophy and skilled in its techniques, who might be lured into ophthalmology to integrate the pathology of the eye with the general science. Ashton accepted the challenge.

And so, in 1948, he came to a nonexisting department in a newly created institute, was given three technicians and one small laboratory, the only furniture of which were the cobwebs of the Moorfields traditions of a century and a half. Today his department occupies an entire floor of the institute and spills over onto another; it houses five full-time pathologists, five part-time research workers, 16 laboratory technicians, three secretaries, and there are usually three or four research workers from abroad, while the reports passing through average some 3,000 each month.

But the immense load of the routine work of a department of this size has not monopolized the energies of this converted pathologist; the bibliography printed in this issue indicates the immense output of original work that he has produced in the last eight years, and the good use to which he has put the rich material that passes daily through his hands. It is to be remembered that it does not include the equally impressive list of work which has issued from his laboratory under his inspiration. This is too well known to require comment. We are all acquainted with his studies on cortisone, on the vascularization of the cornea, on uveitis; with his singularly ingenious and beautiful studies on the anatomy and pathology of the ocular circulation—the uveal vessels, the canal of Schlemm, and the aqueous veins; of his experimental demonstration of the cause and the prevention of retrolental fibroplasia; with his researches on diabetic retinopathy in connection with which he first demonstrated the relationship between the renal and retinal lesions; and with a host of other things. These achievements have earned him a unique place in ophthalmic pathology; in his own country they have brought him the Nettleship Medal (1954), the Middlemore Prize (1955), and the Membership of the Royal College of Physicians of London (1957); and now they have brought him the Proctor Medal.

Stewart Duke-Elder.

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AFTER-DINNER SPEECH

NORMAN ASHTON
London, England

Ladies and Gentlemen:

Here in America I know you think nothing of travelling great distances and gaily hop from state to state, from country to country, from continent to continent and perhaps soon from world to satellite. But in England we are perhaps not yet so fully emancipated in this way and many of us still contemplate even the shortest journey with at least a trace of misgiving inherited from the days of stage coaches and highwaymen.

My usual travel consists of a short ride on a No. 77 bus from Westminster to our Institute in Judd Street, at least it has been a bus since the aqueous flow became blocked in a certain canal. A journey to Wales for instance, would be an event, to Scotland an exploration, and to Ireland—well dangerous.

Ladies and Gentlemen, tonight, I, a Lon-

doner, have come 3,120 miles to dinner! Your hospitality and the meal have been more than excellent, but this is not actually why I came. I am here to show you and to tell you how very deeply I value the tremendous honor you have so generously bestowed upon me.

The Proctor Medal is the most highly prized award in ophthalmologic research in this country today, for its recipient is selected from a group of workers that can boast increasingly fine achievement. Who then would not feel overwhelmed on receiving such an honor? Certainly I do. But it would be foolish to deny my pleasure in this great good fortune or my pride in being the first non-American to receive the award, and I thank you with all my heart for your kindness and I thank you, Dr. Thygeson, for your introduction.

For a few happy days I felt that British ophthalmology had really got one over the net and that it was "our advantage." Then came the news that Dr. Alan Woods had been awarded the Gonin Medal—so then it was "your advantage." He is I understand the first American to receive this international distinction. I would like to take this opportunity of congratulating Dr. Woods—for whom we have a great regard and affection—and also American ophthalmology upon this notable achievement. I only hope that Dr. Woods is not unaware of the exacting condition he must first fulfil—that he must give an address in Lausanne—in French. And here it is my stern duty to warn my successors of the anxieties in store for them, for I feel that they should know that the work for the Proctor Medal really begins after the notification of election. The committee requires a biography, a bibliography, a photograph, a scientific address, comments on receiving the medal and—worst of all—an after-dinner speech. And just in case I should treat this matter lightly I was ominously informed, by way of example, that one of my distinguished predecessors had spoken on the "Origin of the Universe." It seemed to me that anything less than a discourse on the "End of the Universe" would hardly be acceptable. Indeed, standing here at this moment, I feel it has come.

Have you ever tried to write your biography? Born in London, lived in London, still in London. Frankly, I couldn't think what I could have been doing all those years. It seemed that the birth was in order for I found a certificate, fortunately complete in every detail, but from then on there was a pretty unimpressive blank, and the only episodes which kept recurring to me were not entirely appropriate to this occasion.

The photograph—like all photographs—was a shock and to aid the editor had to be clearly marked "this way up," but I still have some fear that it might find its way into the clinical section.

And when my bibliography was complete—in the biggest type we had and spaced out as much as possible—it looked almost rudely brief. Surely I should have done more than this! I must confess that I toyed with the idea of adding a few fictitious references. A title such as "Amplitude of the Z-wave in Aaron rods and incense cones of the Asiatic" 1898, Archives of Egyptian Neurothology, would surely not be checked.

Fortunately this madness passed and as I contemplated my list of publications I began to ask myself, not why I had written so comparatively little, but why I had written at all, or for that matter, why any of us write. And the problem is not a small one. Physicians do it, surgeons do it, some eye chaps do it, and even researchers in their labs can just do it. It has been calculated that nearly one million scientific articles of one kind or another are published each year and it is fascinating to find that scientific journals multiply like bacteria and double their number every 15 years. In 1750 there were 10 scientific journals, in 1950, 100,000 and it is estimated that there will be 1,000,000 in the year 2000. (Most of us fortunately will

be thinking of retiring by that time.) There are some 5,000 medical journals and 3,000 medical books published each year, America being responsible for about a third of the number.

All too rarely do we reflect on the purpose of this vast output, which takes so much of our lives reading it, in order to learn and keep up-to-date, and so much of our time writing it, in order to . . . in order to what . . . that is the question.

My own teacher in pathology, Professor Pulvertaft, once wrote—and he has given me permission to steal from his article—"If you look at any medical weekly, you will notice that the advertisements are always divided into two sections. Those relating to homes for inebriates, night starvation, and quack medicine, are generally at the beginning and end of the issue. Those relating to Frank Pus, M.D., and his friends are in the middle."

It has to be remembered, of course, that this is the only form of medical advertising which is ethically possible and it has gradually developed along several well-defined lines. Let us take removal of the liver, or hepatectomy, as an example.

First, the young house surgeon—inter*nees* do you call them?—reporting his first case does so with an air of apologetic diffidence. He begins like this: "Recovery from hepatectomy in a Mohammedan Siamese twin with acute yellow atrophy and leprosy might possibly be thought of sufficient rarity to justify a brief report." He ends with flowery and profuse acknowledgements to seniors who might be on future election committees.

Secondly, the trainee surgeon who has never had a chance to do a hepatectomy; he speaks in discussions at scientific meetings: "Dr. Frank Pus spoke next. He said he had specialized in hepatectomy and wished to draw the attention of all general practitioners to the great advantage of this operation over removal of the gall bladder—that the patient got more for his money. He had had five thousand of his livers illustrated in colours, he had presented six thousand of his livers to the College of Surgeons and he hoped to see a Hepatectomy Section of the Society of Medicine."

Thirdly, the man who really has done a few hepatectomies. The text consists of a series of blood counts, sections through the falciform ligament, blood chemistry, and radiologic studies "on my last fifty thousand cases of hepatectomy." Sometimes but very rarely "My thanks are due to Drs. Smith, Jones, Brown, and Robinson who did the blood counts, sections, biochemistry, and radiology."

The surgeon or physician who has arrived at an age when dignity is a consideration, develops the scholarly attack. It goes like this "In his Hunterian Lecture entitled 'Hepatectomy and other euthanistic laparotomies' Sir Frank Pus referred to the disembowelling performed on Egyptian mummies as probably the earliest known examples of this operation, although in those preListerian days of course, the subjects were already dead."

A really successful man, however, has a syndrome—(in case there are some here who don't know the meaning of this word, I may remind you of the young nurse who translated it as "A den of vice"). A good syndrome should have two discoverers and three coincidences. Thus—"The Mulligatawny-Pus syndrome. An interesting observation by two well-known hepatectomists working independently is briefly discussed in the current journal of Abysmal Malpraxis of Ruritania. They find that 95 percent of cases of hepatectomy show a right paramedian scar, no excretion of bile, and a resonant percussion note in the right upper abdomen."

So it goes on, until we hear Laud Pus of Pyocyanin drawing attention in the House of Lords to the need of a hepatectomy service in Labrador. Is there then no real medical literature? Yes indeed it is tucked away

at the end of "Synopsis of Current Literature" and it reads as follows: "Bulganotoff states that all operations for complete removal of the liver are fatal within ten minutes."

Of course, on the scientific side we have our gimmicks. For example, take a simple process that everyone imagines they understand—like the sedimentation of red blood cells—dig out the laws relating to spheres falling through space (the more obscure they are the better) and cover pages with formulas and squiggles until you end with an *Index* or *Factor,* which if all goes well should be named after you—like Max Factor. This method will ensure your being invited to all important symposia, because you alone will understand the work. You will have a wonderful time until someone comes along and tries another profitable line, which is the *debunking of well-known work,* and he will point out that the physical laws you have used do not, in fact, apply, because the problem actually relates to bioconcave discs falling through plasma. But don't despair, this will be the beginning of endless articles on the proper interpretation of $_{p1}V$ in relation to $_{p2}V$—but whatever you do, avoid journals on physics.

The other important weapon we laboratory workers have is statistics—which you will remember have been likened to a Bikini —they hide the essentials but reveal interesting possibilities. Now statistics put us "one-up" on the clinician who always tends to imagine that he could do our work equally well if he only had more spare time. A few "correlation co-efficients" and "standard deviations" scattered throughout your paper will soon put that right. It is true that people like Pasteur and Erlich managed to scrape along fairly well without all these significant figures, but they were working only on the prevention and treatment of disease, whereas statistics are much more valuable in that they prevent undesirable pleasures like smoking, drinking, and even eating.

So "getting a job" or "making one's name" are two of the reasons for writing, for certainly we can be sure that medical literature would be much less bulky were all the articles anonymous. But this is why the other fellow writes—we in this room of course have better reasons.

Ideally a scientific paper is an honest open letter to workers sharing the same interests and it should be written simply and directly to its intended readers. Having as its high purpose the advance of knowledge, it should be strictly regardless of personal gain, research grants, and national barriers, and *above all* it should treat the work of others with scrupulous fairness. There is a danger that these ideals may be squeezed out by commercial and journalistic influences and it seems to me that our best hope of maintaining our traditions lies in the example set by members of such excellent associations as your own.

As regards style too often our papers are so complex, and apparently addressed to nobody in particular, that they are almost unintelligible. I always advise trainee research workers to go home and read their papers through to their wives and when they agree that the subject is understandable it is nearing a suitable form for publication. That does not mean of course that scientific articles should begin "Well darling, it's like this . . . ," but that the doctor's wife is an interested and intelligent person, unhampered by medical jargon, and with a woman's wiles can painlessly deliver her husband's "brain-child."

In the world today, however, there seems to be less and less time for perfection. We are in a hurry even when we are lying down. As an example of the absurd position we have reached, take the case of the American business man who went for psychoanalytic treatment. The psychiatrist said "Am rushed for time—can't stop—patients waiting—just lie down on that couch and tell your symptoms to my tape-recorder." Two

hours later when he returned the room was empty but a note on the table read "Rushed for time—can't stop—assignment waiting—have left my tape-recorder talking to your tape-recorder."

We write also I believe because we all fondly hope that our work may have some ultimate application to human needs, although the pure scientist will tell us that the pursuit of knowledge itself is a sufficiently noble purpose and that it is as valuable to know the mating habits of the Drosophila as it is to know the cause of cancer. Of course, researches with no practical bearing on human interests find their immediate justification in satisfying the incessant urge of man to label all things and to discover how they work. But what can be the final value of such efforts? Unless the findings eventually have some use—surely none. I side therefore with Unamuno the Basque philosopher who said "Is it that after all the chief end of man is simply to catalogue the universe? Is it that at last the human race will fall exhausted at the foot of a pile of libraries—in order to bequeath them? To whom? For God surely will not accept them."

Holding these views then, I find it a very great experience to receive the Proctor Medal, for I feel that the work from my unit is accepted as of some value by those most qualified to judge. I know there are many who deserve the honor more, but I would remind them of Fuller's dictum that "it is a worthier thing to deserve honor than to possess it."

I shall carry the medal home to London—if I get it through the customs—as a trophy to share with my colleagues at the institute, all of us the scientific offspring of a great ophthalmologist, a great writer, and a great enthusiast—Sir Stewart Duke-Elder. As I have said on a previous occasion, all of us are entitled to call ourselves Duke-Elderberries.

When you can spare the time to leave your magnificent country for a visit to the old world I can assure you most sincerely that the warmest welcome awaits you at the Institute of Ophthalmology in London.

Institute of Ophthalmology,
Judd Street (W.C.1).

EFFECTS OF TACHISTOSCOPIC TRAINING*

On visual functions in myopic patients

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Introduction

Although the effects of eye exercises and visual training on visual functions have been widely investigated, there have been virtually no studies in which relevant factors have been adequately controlled. Extravagant claims and forthright condemnation have both been based on inadequate evidence and at the same time widespread lethargy concerning the important possibilities of visual training has prevailed.

That the problem is important does not seem to require much justification. Effective vision is a function of learned visual and perceptual habits in a healthy organism as well as of the optical characteristics of the eyes. Most of the training methods advocated have been directed at visual control and perceptual habits. If visual acuity could be increased by training, the effects for myopic patients might be of value not only in civilian life but also in military service.

Claims for visual training have included the successful treatment of eye-strain, myopia, hyperopia, presbyopia, astigmatism, strabismus, and even pathologic conditions, such as cataract and glaucoma. Where a disease exists, the rationale of treatment by exercise methods should be examined critically in relation to etiology and the nature of the disease process. Such considerations are outside the scope of this investigation.

The rationale of a training procedure in cases in which the diminished vision is related to refractive and muscular factors, without observable eye pathology, has seemed reasonable and worthy of experimental evaluation. The present research is concerned with the effects of a training procedure on the vision of myopic patients. The selection of a single category of visual anomaly limits the extent to which the results may be generalized, but has the advantage of greater control of experimental conditions. The tachistoscopic technique developed by Renshaw[1, 47] was employed for training, as described later, and the results with a trained group were compared with those of a control group which received no training.

Research on reduction of myopia

Ophthalmologists have distinguished between structural and pseudomyopia.[2] If the myopia disappears or decreases markedly following relaxation of the ciliary muscle by means of a cycloplegic drug, it is considered pseudo.

Etiology

The etiology of myopia has been a controversial subject. Excessive convergence, excessive accommodation, hereditary struc-

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tural defects, increased pressure of fluids on bending the head, and congestion of the eye coats were postulated in the earliest theories.[3] The list has been greatly enlarged, as shown by Stansbury,[4] to include (1) too short an optic nerve, (2) congenital deficiency of the sclera, (3) disorder of growth, (4) imbalance of the extraocular muscles, (5) psychic and intellectual relation, (6) endocrine dysfunction, (7) avitaminoses, (8) constitutional diseases, (9) biologic variation, and (10) sclerochoroiditis.

Other suggested causes are environmental stress (for example, near work, use of eyes under poor hygienic conditions), drugs, anesthesia, dystrophy of lens fibers, corneal inflammation, circulatory changes, personality defects, variation in normal embryonic processes, prematurity, ciliary spasm, reduction in depth of anterior chamber, disease of anterior segment of eyeball, psychosomatic disorders, and diabetes. Miller[7] proposed that myopia is found where such food elements as fats and other essential foods are lacking in the diet. However, in cases approaching near myopia at birth, the normal amount of fat in the diet will not check the visual axial development because congenital myopia exists.

Crisp's[8] belief is that it may be well to assume that not the single element of heredity, not nutrition alone, not the influence merely of study or other close work, but all three factors jointly, and in varying degree, may be responsible for the anomalous optical measurements of myopic eyes. It might be well to assume that the etiology of myopia varies from case to case.

Refraction

Claims concerning the actual reduction of myopia have produced controversial opinions. Evans[9] has stated that refractive errors are not modifiable by use or abuse and hence are not "curable" by the use of glasses, nor are they modified by "wrong" glasses, eyestrain, "exercise," or other forms of training. If the refraction departs materially (one diopter or more) from the normal curve, he believed that ocular or systemic disease is or has been present, including a radical change of weight.

As an explanation for certain cases in which myopia has been reported as "reduced," Bannon[10] pointed out that a reduction in the correcting lens should not be confused with a reduction in myopia. A reduced correction may only mean that the patient had been wearing an overcorrection for his refractive error.

Certain investigators believe that only low degrees of myopia may be reduced. According to Stoddard[11] myopia greater than 2.0D. is probably mainly anatomic and orthoptics or similar procedures are futile in such cases, whereas myopia less than 2.0D. may be functional or anatomic and the functional or pseudomyopic cases may be less than 1.0D. Stoddard recommends orthoptic or similar procedures routinely when low myopic states exist.

A study by Chance[12] revealed that in those patients exhibiting myopia of less than two diopters in whom the refractive state under cycloplegia was less myopic than without cycloplegia, orthoptic training finally resulted in a reduction of the myopia. But in those cases which showed essentially the same refractive state with or without cycloplegia no improvement in the myopia was obtained by orthoptic procedures.

Olmsted and his associates, according to Chance, Ogden, and Stoddard,[13] suggested that an average of 1.0D. is the amount by which myopia can be physiologically reduced, unless pseudomyopia is present.

The wearing of an undercorrection with base-in prism has also been credited with reducing myopia. Chance and his associates[13] believed that there may be some plausibility in this theory. Henderson was reported by these authors to have shown that excitation of the ciliary body by the parasympathetic nerves with reciprocal inhibition of the sympathetic nerves causes accommodation, or a relative myopia, with respect to the basic refractive state. But excitation by way of

the sympathetic with reciprocal inhibition of the parasympathetic results in a relative hypermetropia with respect to the basic refractive state. The basic refractive state is defined as the refractive state in which only normal autonomic tone is present.[13]

Chance et al.[13] suggested that what has been thought to be a simple relaxation of accommodation in the change of ocular focus from near to distance fixation is really a reciprocally innervated dynamic change in the refractive state. According to their report, Morgan, Olmsted, and Watrous showed that the change resulting from parasympathetic excitation is of the order of 10 diopters and that from sympathetic excitation is approximately 1.0 diopters, while Henderson showed that this finding is largely due to the difference in mechanical advantage of the ciliary muscle fibers innervated by the two branches of the autonomic nervous system.

Drucker[14] is of the opinion that concave lenses themselves might be a cause of progressive myopia, since he noted that myopic patients who refuse to wear their minus corrections constantly appeared to suffer less from progressive myopia than did those who wore their minus corrections most faithfully. Drucker cited his own personal experience. At one time he required a spherical minus correction of almost two diopters, and had a visual acuity of below 20/200. By wearing plus lenses with base-in prisms for reading, progressively increasing the plus until two diopters could be worn for near, his visual acuity gradually improved to 20/40 and objective testing indicated a residual myopia of 0.5D.

Rasmussen[15] has stated that "myopia is a shifting of the eye's optical focus forward of the retina through displacement and distension of the crystalline and cornea by binocular close-visual maladjustments." He applied this theory to 125 consecutive clinical cases in the National Health Service without selection. Cases of marginal ametropia were eliminated from the study, leaving 86 cases with myopic spherical or spherocylindrical correction in all meridians of from 0.5D. to 12D. The method was to reduce the spherical correction only for close vision and to do so only by maintaining a properly balanced relation between convergence and accommodation. In general, the rationale of his technique was to establish parallel rays or "infinity" at distance (regardless, or in spite of, whether the acuity corresponded). For the 86 cases, ranging in age from nine years to 65 years, the average manifest myopia was 2.75D. and the average reduction was 1.68D.; the average presbyopic difference was 0.54D., leaving an average net reduction of 1.14D. For the 59 cases under 40 years of age, the average manifest myopia of 2.58D. was reduced by 1.45D. or 56 percent.

Muscle balance

The frequent association of muscle imbalances and myopia has led Drucker[14] to suspect that there may be a relationship which may account for certain cases of apparent reduction in myopia.

In a statistical study of functional muscle tests in axial myopia, Snell[16] found nearly twice as much esophoria as exophoria. In 1,078 cases of myopia, esophoria was exhibited in 55.3 percent and exophoria in only 30.4 percent of the cases.

In Rasmussen's study[15] the incidence of esophoria was over three and a half times that of exophoria, but only twice as much in the older age groups (51 to 65 years). He believes that this is good evidence that esophoria is a functional error largely associated with myopia through the development of overconvergence from very early maladjustments.

Visual acuity

Increased ability to see following visual training has been attributed to a number of factors. Among these are training in the interpretation of blurred images, improved visual memory, and elimination of poor visual habits. However, uncorrected visual

acuity is not an accurate indicator of the degree of myopia. Rubin et al.[17] gathered data on 1,105 eyes showing simple myopia as determined subjectively by the fogging technique. All had normal acuity when corrected. Errors varied from 0.25D. to 3.5D. There was much overlapping in naked acuity, especially where vision was reduced to approximately 20/200. This was attributed in part to the few letters available for testing in this range.

Lancaster[18] cited the case of a young man who had been wearing concave lenses and asked if there was any way he could pass the test for 20/20 vision. Vision was 20/15 with glasses but was 20/30 without glasses. He was given a +1.0D. sph. for each eye to wear constantly for three days. His visual acuity was 20/15 without glasses and 20/15 with a +0.5D. sph., and he read some letters of the 20/20 line with a +1.0D. sph. His myopia was not cured because he did not have myopia to begin with. He learned to relax his accommodation. He was judged to be myopic by some one because when a −0.5D. sph. was placed before his eye, he said, "that is better, clearer." That a young man with 20/15 vision sees better with a −0.5D. sph. shows that he has good accommodation, not that he has myopia.

In cases of pseudomyopia vision is variable. This type of myopia is considered identical with spasm of accommodation and a factor in causing progressive myopia. Spencer-Walker[8] inferred that poor vision is a stimulus to spasm of accommodation. This author suggested that there may be many myopes in schools and classes for the partially seeing who suffer from accommodative spasm, suppression, and so forth. Although they benefit from the visual rest-negative treatment, Spencer-Walker believed they would benefit still more from positive orthoptic treatment.

In two experiments performed by Marg[19] some hundred clinic patients between 14 and 40 years of age were measured for visual acuity wearing their newly determined prescription combined with a +3.0D. sph. lens before each eye. Then, after instructions on how to see more clearly followed by a short practice period, they were again measured. Only one subject of the hundred demonstrated unusually good transient acuity (a flash) but she was unable to maintain it or repeat it for measurement of refraction. The next experiment consisted of five specially selected subjects who could flash. Some of them were undergoing Bates' training at the time. Visual acuity was improved from around 20/200 to 20/50. The refractive state of the eye was measured by skiametry at one time and with the coincidence optometer of Fry at another. No change in power was found by skiametry from normal to flash vision. The optometer indicated changes of −0.22D. to +0.27D. none of which was significant at the 95-percent confidence level. For the change in acuity to be attributable to negative accommodation, the dioptric change would need to be from about plus 1.0 diopters to 2.5 diopters, depending on the subject.

Visual fields

Renshaw[1] has shown that tachistoscopic visual form training may result in a significant and large increase in the form-field of the two eyes. The form-field is defined by the solid angle within and beyond the region of the anatomic macula in which an observer is able to distinguish shapes.

Reading

The importance of reading in regard to myopia is fully appreciated when we realize that near work has been and still is considered an important cause of myopia by many investigators. For example, Sondermann[20] recommends the avoidance of repeated eye movements in reading by turning the head. Because, in reading, the lines are changed up to 2,000 times an hour, an hereditary weak posterior scleral segment may become enlarged.

The relationship between visual training and reading as well as academic improve-

ment has been studied by Olson et al.[21] In their investigation 65 students accepted the dean's suggestion that they participate in this study; most were C students or worse. These were divided into four matched groups: (1) visual training group; (2) visual training and counseling group; (3) counseling; (4) control group. Forty-nine completed the program. Visual training groups showed reading gains significant at the one-percent level and maintained gains after summer vacation. Improved comprehension, academic improvement, and better patterns on the Bernreuter Personality Inventory were indicated but the results were not statistically significant.

In another study, 75 children were given tachistoscopic training twice weekly, and their scores on school achievement and intelligence tests were compared with those of a control group receiving no special training.[22] Average grades on reading tests were higher in the trained than in the untrained group.

Visual training

Lancaster[18] reasoned that seeing involves ocular and cerebral factors, and, therefore, visual training may be able to improve the cerebral factor because such training includes repetition, practice, and learning.

More recently, Lancaster[23] expressed the belief that improvement in myopia may be due to learning how to interpret blurred images. Kratz[24] believed that only when myopia is associated with small degrees of astigmatism, excessive close work, or neurasthenia and muscle imbalance can physiologic improvement be expected from visual training procedures.

Duke-Elder[25] and many others have thoroughly denounced the Bates' method of training. According to Cowan[26] it has never been shown that the onset or arrest of typical myopia or any other refractive error can be prevented. He believed that "we have no more means of controlling the growth of the eyeball than we have of regulating the growth of any other feature of the body."

Visual training methods are legion. In addition to the methods advocated by Bates[27-29] and Peppard,[30] well-known training methods include: orthoptic training, tachistoscopic training,[31] daily reading of the smallest letters on a Snellen test chart,[32] use of a vectoluminator with polarized targets for the two eyes,[33] projected moving patterns produced by a kaleidoscope,[34] recognition of puppet positions for training athletes,[35] use of stereoscopic slides and pointers,[36] the Skeffington[44] technique, and others.[37-42]

Some investigators stress that pseudomyopia may respond to visual training[10, 11] because it is due to excessive accommodation. However, medical and scientific opinions concerning true myopia are typified by Post who, in discussing a paper by Lancaster,[18] stated that while visual acuity in myopia may be improved appreciably by exercises or training, in his experience there was no evidence of a significant change in the ametropia.

Although visual training may produce improvement, it may be temporary, necessitating periodic repetition. In one case, cited by Preble,[43] vision in a co-operative young girl, aged 13 years, improved from 20/200 to 20/30 in both eyes. However, vision of 20/30 apparently required too great an effort and the initial vision of 20/200 was more satisfactory to the patient. In addition, while the vision improved, the myopia increased from 2.0D. to 3.0D. on retinoscopic examination.

Training

Sloane, Dunphy, and Emmons[44] co-operated in investigating the effect of a simple group training method upon myopia and visual acuity. Eight boys ranging in age from 14 to 18 years were given group training. A three-dimensional tachistoscope was used. Sessions were held for seven consecutive weeks, omitted for three weeks (during a school vacation period) and resumed for a final five-week period. Each training ses-

sion lasted one-half hour. There was some slight variation in the results of the vision tests made by the three examiners. In each case the value agreed upon by the three examiners as the best estimate of the subject's preliminary and final vision was given. When two of the three examiners obtained identical results, that value was chosen; when none of the three obtained the same result, a value approximately midway between the extremes was selected as being the best estimate. No cycloplegic was used for refracting.

Six of the boys had exactly the same assigned value before training, and five obtained the same value, after training, on one chart as they did on the other. The two who varied before training did so only in a slight degree; and the same was true of the three who showed some difference in response to the two charts after training. In no instance did these estimates vary more than "one line" on the charts.

None of the subjects showed a decrease in degree of myopia in both eyes on retinoscopic values assigned after training. Two boys had a decrease in one eye, and in all the others there was a slight increase in myopia. The changes in myopia were small and many were within the limits of error and of an individual's daily variation in refractive error.

Hildreth et al.[45] studied the effect of visual training on myopia. Fifty-four patients were selected from a group of 84 receiving visual training as a substitute for glasses. All had myopia ranging from 1.0D to 3.0D. The results were observed for a period of more than a year. The exercises were given by a group of optometrists. The patients were carefully examined and observed before and after training by a group of ophthalmologists from the staff of the Washington University. The training consisted of three sequences of six to 12 steps involving approximately one hour daily for one month. It included the fixation of targets, at different distances, monocular and binocular, moving and stationary, and stereoscopic.

Thirty (55.5 percent) of the 54 selected cases revealed no change in their visual acuity, while 12 (22.2 percent) showed a definite improvement, the best results being obtained in the cases with a small degree of myopia. In 12 patients (22.2 percent) there was so slight a change that they were excluded from this group. Eleven of the improved patients were rechecked at an interval of 15 to 23 months after completion of the training. Five retained their improvement, while one showed less acuity than before training.

During the training no change was observed by the supervisory ophthalmologists in the retinoscopic refraction nor in the ophthalmoscopic appearance. Slight differences in muscle balances were occasionally noted. The examining ophthalmologists concluded that visual training can only be of temporary value in myopes whose visual acuity is lower than suggested by the degree of their myopia. The improvement is practically always only temporary and is due to improvement in visual memory.

In the Wilmer Institute study,[46] 103 uncomplicated myopic subjects were selected for visual training. Both visual acuity and refractive error were measured before and after the training program. Thirty subjects showed an improvement on all four of the visual charts used, with a 27-point increase on the percentage visual acuity; 31 subjects did not improve on all charts but showed a decrease of 10.8 points. Woods concluded that the degree of average improvement was within the limits of errors of measurement by the subjective test of visual acuity and that the results indicated the improvement to be based on the ability to interpret blurred images rather than on any change in refractive error.

Discussion

The foregoing discussion of the literature presents many conflicting facts and opinions.

Nevertheless, a number of reputable investigators have reported improvement in visual acuity, increased size of visual fields, reduction of refractive error, and improvement in reading in some myopic patients as a result of treatment by prescription of corrective lenses or by various training methods.

Prevailing medical opinion, supported by the findings of Sloane, Dunphy, and Emmons,[44] Hildreth et al.,[45] and Woods,[46] and by the expressions of Lancaster[18] and Post,[18] is reluctant to accept the reduction of myopic refractive error by these methods, but is more amenable to accepting competent evidence demonstrating improvement of visual acuity, size of visual fields, and reading, since these are presumed to depend to a large extent on learning. Nevertheless, the results reported have not demonstrated spectacular success, even for these functions.

The evaluation of these studies is difficult, first, because none of them has made use of a control group, which would provide a proper estimate of a base rate against which to compare the effects of training; and, second, because in most cases statistical tests of the significance of observed differences were not presented, nor were the data presented in such form that they could be computed. It can only be reported that various training methods were used with subjects of varying age, varying motivation for the training, and varying degrees of myopia, and that in a proportion, varying from about 20 to 30 percent, improvement in visual acuity of varying degrees was found; in a comparable proportion of subjects, increased myopia resulted. In only one investigation, by Hildreth et al.[45] was a follow-up over a time made and this showed that 45 percent of 11 cases retained their improvement, while only one subject had regressed lower than the pretraining level.

The present investigation was designed, as far as possible, to overcome the limitations of previous work in this area, although circumstances precluded inclusion of a follow-up study. The research design specified the characteristics of subjects to be included, the uniform training procedure to be followed, and provided for a control group. The analysis of results made use of quantitative methods of treating the various measures and tests of significance of the differences obtained between experimental (trained) and control (not trained) groups of subjects.

The limitations of this study include several that were planned and a few unintended ones which were the inevitable result of the difficulties of obtaining subjects with the desired characteristics. It was planned to use only structural myopes, without complicating pathologic process, and to use only the Renshaw tachistoscopic training technique for training. The advantages in experimental control, gained with these limitations, were judged to offset the restrictions imposed on generalization of results. It was also intended to utilize for both experimental and control groups, subjects who would be highly motivated to improve their vision. As discussed below, there was evidence of a substantial difference between the two groups in this respect which may account, at least in part, for the results obtained.

Selection of subjects

The experiment was carried out with 140 subjects. Of these, 80 received tachistoscopic training and 60 were given only the initial and final visual evaluation, but no training. The trained group will be referred to subsequently as the Experimental Group and the nontrained group, as the Control Group.

The criteria for selection of subjects, as specified in the original plan of this project, were:

1. *Age.* Subjects were to be drawn from the range of 10 to 30 years of age, spanning generally the periods of schooling and most active military service;

2. *Type and degree of ametropia.* Subjects were to be accepted only if they had between 0.5D. and 3.5D. of myopia, as determined under cycloplegia; with not more than 2.5D. of astigmatism; not more than

2.0D. of anisometropia; with correctible visual acuity of 20/20; and if they had no complicating internal or external pathologic condition.

Sources of subjects

Initially, subjects were obtained from the clinics of the New York Eye and Ear Infirmary, the Mary Hitchcock Hospital, Hanover, New Hampshire, and from private practice. In order to recruit a larger number of subjects than were available for training classes from these sources, an appeal for volunteers was broadcast over a radio program in New York. The announcement stated that The Ophthalmological Foundation, Inc., with the co-operation of the New York Eye and Ear Infirmary, was undertaking research on visual training for myopic patients under 21 years of age, with refractive errors of −3.0D. or less. It was explained that the training course would consist of three half-hour sessions per week for a period of 10 weeks. Eligible persons were invited to apply to the foundation office.

Most of the subjects were obtained through this appeal. Applicants were given preliminary screening by the office staff and accepted or rejected after the first ophthalmologic examination. Subjects accepted for training were required to agree to attend regularly. They were informed that if their attendance during the first two weeks of training was unsatisfactory, they would be dropped from the training classes, but would still be required to return later for another ophthalmologic examination; that is, cases dropped from the experimental group for unsatisfactory attendance (reflecting lack of interest or inability to attend) were to be held for the control group. The motivation of most of the subjects who entered the training classes was intense. There were many enthusiastic expressions of hope to improve their vision and "discard their glasses" after completing the course.

Although obtained from the same general source, the motivation of the control group was, by contrast, generally poor. This group included 20 individuals who were dropped from the training classes because of poor attendance, and who agreed to return later for final visual evaluation. It also utilized 40 applicants who were informed that they were too late for the current training classes, but would receive priority in later classes if they participated as control cases in the current experiment and took the visual examinations.

Considering the circumstances of their recruitment, it may be inferred that the experimental group, for the most part, manifested intense motivation to improve, while the motivation of the control group was questionable. This interpretation is supported by the fact that the staff experienced considerable difficulty in inducing the patients in the control group to return for their final examinations, and the interval between initial and final examinations for the control group is significantly longer than that of the experimental group. This difference between the two groups was not intended, but should be considered in evaluating the experimental results.

Characteristics of subjects

The total sample of 140 subjects had a slight majority of females, 55 percent. They ranged in age from six to 47 years and in education from the first grade to college graduate. Forty-five percent were students, 11 percent housewives, and 34 percent were employed in various occupations. Occupation was not recorded for the remaining 10 percent.

The experimental and control groups are quite well matched with respect to these characteristics, as shown in Table 1. The means and standard deviations* are similar

* Explanation of terms. The mean refers to the arithmetic mean or average of the distribution and provides a measure of central tendency. The standard deviation is a measure of dispersion of cases around the mean and refers to the range, in units of measurement, above and below the mean, which includes the middle 68.26 percent of the cases.

TABLE 1

COMPARISON OF EXPERIMENTAL GROUP AND CONTROL GROUP WITH RESPECT TO POPULATION CHARACTERISTICS

		Experimental Group	Control Group
Number of cases		80	60
Age (yr.)	Mean	22.75	22.32
	S.D.	10.01	9.71
Education (highest grade completed)	Mean	10.60	10.59
	S.D.	3.47	3.91
Proportion of females in group		54	56
Occupation	Student	35 (44%)	28 (47%)
	Housewife	9 (11%)	6 (10%)
	Other (employed)	26 (33%)	21 (36%)
	Not recorded	10 (12%)	4 (7%)

and the proportion of each sex and distribution by occupation, within the categories recorded, are closely comparable.

The mean age of each group is between 22 and 23 years, with a standard deviation of 10 years. The mean educational level is between the 10- and 11-year level. This is considerably lower than the level expected for the mean age reported. However, 40 percent of the experimental group and 38 percent of the control group were over the age of 22 years, the normative age for college graduation. If it were assumed that all subjects above 22 were college graduates and that all other subjects had achieved schooling expected for their ages, the median education for both groups would be between the 15- and 16-year levels. Hence, it may be concluded that the present samples are below average in educational level, although comparable with each other. This educational retardation is consistent with the fact that they were drawn from the lower economic and social strata of the general population.

VISUAL CHARACTERISTICS

The visual characteristics of the two groups are summarized in Table 2. It will be seen that they are closely matched in degree of myopia, anisometropia, and visual acuity. Refractive error was determined by retinoscopy under cycloplegia, and is reported in terms of spherical equivalent. The mean refractive error for the experimental and control groups, respectively, is −2.27D. and −2.31D.; the variances are approximately comparable. Anisometropia was computed as the difference between the spherical equivalent of the two eyes. The means, in diopters, for the two groups are 0.41 (experimental) and 0.45 (control); the standard deviations of both are 0.49. Visual acuity, with correction, was measured for right eye, left eye, and both eyes, separately, on three visual acuity charts. These were charts designed by Berens, Classon, and Ferree, and Rand. The visual acuity fractions were converted to decimals to facilitate statistical analysis. The decimal scores for each eye on each chart, of the combined experimental and control groups, were transformed to T* scores with a mean of 50 and a standard deviation of 10. The mean visual acuity T scores for the experimental group, with correction, are O.D., 49.23 (20/25); O.S., 50.05 (20/24); and O.U., 50.55 (20/21). For the control group, the comparable scores are O.D. 50.31 (20/23); O.S., 51.0 (20/23); and O.U.,

*A T scale is a statistical technique of transforming measurements into standard units for convenience of computation and uniformity of units. The mean score of a T scale is arbitrarily fixed at 50 and each 10 points of score above or below are equal to one standard deviation.

TABLE 2

COMPARISON OF EXPERIMENTAL AND CONTROL GROUPS WITH RESPECT TO VISUAL CHARACTERISTICS

Variable		Experimental Group	Control Group
No. cases		80	60
Refractive error[1]	Mean	−2.27D.	−2.31D.
	S.D.	1.47	1.05
Anisometropia[2]	Mean	0.41D.	0.45D.
	S.D.	0.49	0.49
Visual acuity cc[3]			
O.D.	Mean	49.23 (20/25)	50.31 (20/23)
	S.D.	8.04	7.50
O.S.	Mean	50.05 (20/24)	51.00 (20/23)
	S.D.	9.02	7.88
O.U.	Mean	50.55 (20/21)	50.74 (20/21)
	S.D.	8.23	7.11

[1] Spherical equivalent of refraction determined by retinoscopy under cycloplegia.
[2] Computed as absolute difference, regardless of sign, between spherical equivalents of refractive errors of the two eyes.
[3] Average of T score equivalents for three charts: Berens, Classon, and Ferree-Rand.

50.74 (20/21). The variances of the experimental group are slightly greater than those of the control group.

From these data it is apparent that the selection of subjects, according to the criteria stated earlier, was reasonably good and that the two groups were well matched in their visual characteristics. The range of myopia in both samples was greater than originally planned, but the restrictions with reference to anisometropia, astigmatism, pathology, and correctible visual acuity were strictly observed.

PROCEDURE

The effect of visual training on myopia was evaluated by comparing the differences between initial and final measurements, with an interpolated period of tachistoscopic training in the experimental group, with the corresponding differences between initial and final measurements of the control group, which received no training and had only an interpolated period of time. This design is less adequate than one in which some form of placebo is given to the control group. However, it was impossible to contrive a suitable regimen which could serve as a placebo, and the interpolated period of time, during which the control cases followed their normal daily routines, was adopted as the best available substitute.

PRETESTS

The initial examination, given to all subjects, included the following performed by ophthalmologists.*

a. History, primarily ocular, medical, personal, and family.

b. Examination of the eyes, including external, media, fundi, size, and reaction of pupils, and so forth.

c. Vision, uncorrected, O.D., O.S., O.U., under controlled illumination, on three charts: Snellen letter, Berens, and Ferree-Rand. In addition, the Classon chart,† with timed exposure, was conducted by means of mounting a shutter on the regulation Classon

* The following ophthalmologists co-operated in performing these examinations: Hanford L. Auten, Jr., G. Calhoune, S. Chamichian, Gerald Fonda, Louis J. Girard, Jerry Jacobson, F. Jones, and Frederick K. Reid.

† Designed for the project by Dr. Louis J. Girard.

projector. After conducting the test with unlimited exposure, letters of this visual angle were exposed at diminishing speeds until the minimum exposure to read individual letters of each angle was determined.*

d. Refraction by retinoscopy, with and without cycloplegia; manifest and cycloplegic refraction, near vision, and amplitude of accommodation.

e. Visual acuity determined as in (c) above, with best correction.

f. Motility studies, including phorias and fusional amplitudes at distance and near, nearpoint of convergence, and versions.

g. Reading speed test, speed and comprehension on Minnesota Speed of Reading Test, Form A for adults and appropriate tests for school-age children.

h. Retinal rivalry rate, as determined by Renshaw retinal rivalry slide; the eyes are dissociated by polaroid glasses and the rate of alternation of retinal dominance determined for a period of one minute.

i. Visual form field, determined for each eye on the Ferree-Rand perimeter, using a 20-point test letter E. The form field was determined subjectively, the test letter being brought centripetally toward the fixation point until the direction of the letter was accurately reported. Determinations were made for the 0, 90, 180, and 270 degree meridians and the findings reported on a field chart.

Training

The tachistoscopic technique for discrimination of form of distant test objects developed by Samuel Renshaw[47] was the basis of the training course. This method is adapted to group training and has the advantage that it is free from criticism with reference to its influence on dioptric and neuro-muscular anomalies.

After completion of the pretest examination, subjects in the experimental group were assigned to classes which met for 30-minute training sessions, three times per week for 10 weeks. These classes were conducted by a trained assistant* under the supervision of the ophthalmologists responsible. Classes were arranged, as far as possible, in homogeneous age groups.

The procedure outlined in the manual on *Tachistoscopic Procedure of the Three Dimensional Tachistoscope for Far-Point Training* by Samuel Renshaw[47] was followed without deviation. One of the investigators† consulted with Dr. Renshaw at Ohio State University and incorporated his suggestions concerning the training technique.

A detailed account of the training technique may be obtained by reference to the manual.[47] In brief, it involves exposure of groups of digits, with progressive increase of number of digits and decrease of exposure time. A multiple digit slide is projected which provides 50-point test objects on the screen. The subjects seat themselves at a distance from the screen where the digits are clearly recognized without squinting. Each subject measures and records his own distance from the screen to his seat. Training starts with three-digit slides at an exposure of $\frac{1}{25}$ of a second, and, as the training progresses, the span of the digits is increased and the time exposure decreased until nine digits at $\frac{1}{100}$ of a second is accomplished. When the subject consistently records the digits correctly, he is progressively removed a foot further from the screen.

After demonstration of the tachistoscope, a projector with an Alphax shutter attachment, the subjects were arranged before the screen in positions such that all could see the exposures conveniently. Those with more moderate myopia were encouraged to work without correction, moving closer to the screen, if necessary, and moving away gradu-

* At the time this study was conducted, there was no agreement on the best form of visual acuity test within the Armed Forces Vision Committee.

* Miss Patricia Rainier was the assistant; Dr. Conrad Berens, Dr. Louis J. Girard, and Dr. Hanford L. Auten, Jr., supervised the training.

† Dr. Louis J. Girard.

ally to 20 feet as the training progressed. Subjects were urged to make a genuine effort to improve. Training sessions were conducted informally with a pause between each exposure for checking results. At the end of each session the work of each subject was checked for results, personal comments, and answering questions.

Posttests

With the exception of the history, the entire schedule of pretests was performed again, by one of the co-operating ophthalmologists, after completion of the 10-week training course, for the experimental group, and after the expiration of 10 weeks for the control group when this was possible.

Results

Time elapsed between pre- and post-examinations

As indicated previously, it was intended to obtain posttraining examinations for experimental cases at the conclusion of training and for the control cases after a similar period of time. However, principally because of their lesser interest in the project and difficulties in scheduling, the average period for the control cases was 16 weeks (mean of 111 days), in contrast to 12 weeks (mean of 83 days) for the experimental group. The mean and standard deviation of number of days between pre- and postexaminations for both groups are presented in Table 3. As indicated by the probability value (p less than 0.01) in Table 3, a difference of the magnitude obtained between group means in this study would occur by chance variation alone less than once in 100 occasions. Hence, this difference is considered, statistically, highly significant.

TABLE 3

Time elapsed between pre-examinations and postexaminations

Variable	Experimental Group		Control Group
No. of cases	80		59
Mean no. of days	82.99		111.09
Standard deviation	32.50		59.11
Mean difference (da.)		28.10	
t		3.47	
p		<0.01	

Visual acuity

Visual acuity measures were converted to decimals and transformed to T scores, as already described under "Visual characteristics of subjects," to facilitate quantitative treatment of data. The summary statistics reported in Table 4 represent the average T scores of corresponding measures on the Berens, Ferree-Rand, and Classon charts. Although the decimal and conventional fraction equivalents of the T score means are reported in Table 5, the data are here given in T score units in order to present the full statistical analysis of all group comparisons on the visual acuity measures. The direction and relative magnitude of shifts from pre- to postmeasures in both groups and in the various specific conditions measured is of considerable interest.

Table 4 compares pre- and post-mean acuity for O.D., O.S., and O.U., both with and without correction, for the experimental and control groups, separately.

The striking impression one receives, on observing that all the differences in visual acuity reported for the experimental group are positive and all those for the control group negative, is increased when it is discovered also that all differences reported in Table 4 are highly significant statistically.*

These results show that the experimental group improved, after an interpolated period of tachistoscopic training, to a highly significant degree, whereas the control group deteriorated in like manner. Of the 80 experimental cases, 74 improved, two remained

* The probability values, marked with a double dagger (‡, p less than 0.001) indicate that differences of the magnitudes obtained in these comparisons would be expected to occur, by chance variation alone, less than once in 1,000 occasions.

TABLE 4

VISUAL ACUITY RESULTS: MEANS AND STANDARD DEVIATIONS OF VISUAL ACUITY T SCORES FOR EXPERIMENTAL AND CONTROL GROUP, PRE-EXAMINATIONS AND POSTEXAMINATIONS: EACH EYE AND BOTH EYES, WITH AND WITHOUT CORRECTION[1]

Experimental Group		Pre-T Scores		Post-T Scores		Mean Difference
	N[2]	Mean	S.D.	Mean	S.D.	
O.D.sc	80	48.76	9.06	54.80	8.76	6.04‡
O.S.sc	80	49.44	8.67	55.24	8.75	5.80‡
O.U.sc	76	49.45	8.97	55.21	8.68	5.76‡
O.D.cc	75	49.23	8.04	55.67	7.90	6.44‡
O.S.cc	75	50.05	9.02	55.36	8.03	5.31‡
O.U.cc	71	50.55	8.23	55.66	7.55	5.11‡
Control Group						
O.D.sc	60	50.10	9.24	45.83	9.73	−4.27‡
O.S.sc	60	49.43	9.22	45.25	9.45	−4.18‡
O.U.sc	59	49.68	8.62	45.27	9.42	−4.41‡
O.D.cc	58	50.31	7.50	44.31	6.83	−6.00‡
O.S.cc	58	51.00	7.88	43.60	6.86	−7.40‡
O.U.cc	57	50.74	7.11	43.58	6.70	−7.16‡

‡ $P < 0.001$.

[1] Eighteen visual acuity T scores, with Mean = 50 and S.D. = 10, were obtained for the combinations of two test conditions (with and without correction worn), three eye conditions (O.D., O.S., and O.U.) and three charts (Berens, Ferree-Rand, and Classon). For each T score distribution, the decimal equivalents of pre- and postacuity fractions of both groups were pooled. The scores reported in this table are average T scores for the three charts.

[2] Numbers of cases for different measures in this and subsequent tables vary slightly because of clerical errors in initial recording of data.

unchanged, and four decreased from pre- to posttesting, while of the 60 controls, one increased and 59 decreased to some extent.

The mean differences in average T scores, in the right-hand column of Table 4, may be further compared in terms of amount of change. The average change for the experimental group, without correction, was +5.87; with correction it was +5.62. For the control group, the corresponding averages were, without correction −4.29 and with correction −6.85. These differences are approximately the same, although in reversed direction for the two groups.

Table 5, which shows the decimal and visual acuity fraction equivalents of the average T scores reported in Table 4, permits an evaluation of the changes in conventional terms. From these data it may be seen that the experimental group improved, on the average, from 20/125 to 20/77, monocularly, and from 20/98 to 20/63, O.U., uncorrected, and from 20/25 to 20/21, monocularly, and 20/21 to 20/19, O.U., corrected. On the other hand, the control group changed from 20/115 to 20/157, monocularly, and 20/97 to 20/131, O.U., uncorrected, and from 20/23 to 20/31, monocularly, and 20/21 to 20/28, O.U., with correction.

REFRACTIVE ERROR

Refraction by retinoscopy under cycloplegia, obtained by the co-operating ophthalmologists, was converted to spherical equivalent for computational purposes. The results for the two groups are summarized in Table 6.

These results are consistent in direction of change with those for visual acuity. Both groups experienced a small, but statistically significant, change. The experimental group improved approximately one fourth of a diopter (+0.22) and the control group deteriorated approximately the same amount (−0.29). However, in terms of individual cases, the changes in refractive error were not as striking as those for visual acuity. Of the experimental group, 69 percent improved, 18 percent remained unchanged, and 13 percent decreased. On the other hand, 79

TABLE 5

DECIMAL AND VISUAL ACUITY FRACTION EQUIVALENTS OF T SCORE MEANS AND DIFFERENCES

Variable		Experimental Group			Control Group		
		O.D.sc	O.S.sc	O.U.sc	O.D.sc	O.S.sc	O.U.sc
T score	Pre-	48.76	49.44	49.45	50.10	49.43	49.68
	Post-	54.80	55.24	55.21	45.83	45.25	45.27
	Diff.	6.04	5.80	5.76	−4.27	−4.18	−4.41
Decimal	Pre-	0.156	0.165	0.204	0.181	0.166	0.206
	Post-	0.253	0.274	0.319	0.127	0.127	0.153
	Diff.	0.097	0.109	0.115	−0.054	−0.039	−0.053
Fraction	Pre-	20/128	20/121	20/98	20/110	20/120	20/97
	Post-	20/79	20/75	20/63	20/157	20/157	20/131
		O.D.cc	O.S.cc	O.U.cc	O.D.cc	O.S.cc	O.U.cc
T score	Pre-	49.23	50.05	50.55	50.31	51.00	50.74
	Post-	55.67	55.36	55.66	44.31	43.60	43.58
	Diff.	6.44	5.31	5.11	−6.00	−7.40	−7.16
Decimal	Pre-	0.795	0.831	0.950	0.854	0.857	0.929
	Post-	0.960	0.975	1.057	0.675	0.625	0.705
	Diff.	0.165	0.144	0.107	−0.179	−0.232	−0.224
Fraction	Pre-	20/25	20/24	20/21	20/23	20/23	20/21
	Post-	20/21	20/21	20/19	20/30	20/32	20/28

percent of the control group decreased, 11 percent were unchanged and 10 percent improved to some extent.

ANISOMETROPIA

An estimate of anisometropia was obtained in terms of the absolute difference, in diopters, between the spherical equivalents of the two eyes, regardless of sign, at each examination. The mean and standard deviation of these difference scores, and the corresponding medians and modes of the distributions of the two groups, for pre- and postexaminations, are reported in Table 7.*

* The mean, or arithmetic mean, is the weighted average of all scores and is the most commonly used measure of group central tendency. However, the mean is an accurate measure of central tendency only when the distribution of scores is symmetric. In the present case, the distribution of anisometropia scores is not symmetric, but skewed, with a considerable pile up at zero. Accordingly, two other measures of central tendency

TABLE 6

REFRACTIVE ERROR RESULTS: MEANS AND STANDARD DEVIATIONS OF SPHERICAL EQUIVALENT FOR EXPERIMENTAL AND CONTROL GROUPS, PRE- EXAMINATIONS AND POSTEXAMINATIONS, FOR O.D. AND O.S., SEPARATELY

Variable		Experimental Group		Control Group	
		O.D.	O.S.	O.D.	O.S.
No. of cases		70	71	58	59
Pre-examination	Mean	−2.27	−2.22	−2.33	−2.42
	S.D.	1.47	1.33	1.05	1.05
Post-examination	Mean	−2.03	−2.03	−2.61	−2.72
	S.D.	1.42	1.26	1.15	1.13
Difference		+0.24	+0.19	−0.28	−0.30
t		5.54	4.35	7.15	7.10
p		<0.01	<0.01	<0.01	<0.01

TABLE 7

ANISOMETROPIA RESULTS: MEANS AND STANDARD DEVIATIONS, MEDIANS AND MODES OF ANISOMETROPIA ESTIMATES FOR EXPERIMENTAL AND CONTROL GROUPS ON PRE- AND POST-EXAMINATIONS

Variable	Experimental Group		Control Group	
	Pre-	Post-	Pre-	Post-
No. of cases	70	70	58	58
Mean	0.41	0.39	0.45	0.44
S.D.	0.49	0.50	0.49	0.51
Median	0.25	0.25	0.25	0.25
Mode	0.0	0.0	0.0	0.0

The distributions of anisometropia scores are skewed toward the zero extreme. However, it is apparent from Table 7 that anisometropia was not affected by the tachistoscopic training.

FORM FIELD

Size of form field was estimated for each eye by measuring the four radii at 90- and 180-degree angles on the perimeter chart. The results, in degrees, representing the average of four radii, are summarized in Table 8.

Although the pretest form fields of the control group are slightly higher, the differences between the means are not significant. The results, with regard to the effects of training, on the other hand, are highly significant and follow the pattern already seen with visual acuity and refractive error. The form fields of the experimental group increased, on the average, and those of the control group decreased. The improvement of the experimental group (average of two eyes =10.52) is greater in magnitude than the decrease of the control group (average of two eyes =4.16). The same trend appears in an analysis of changes in individual cases. Of 75 experimental cases, 72 increased, one was unchanged, and two decreased, while of 56 control cases, 49 decreased, one was unchanged, and six increased.

READING SPEED

Reading test scores were analyzed only for subjects between the ages of 11 and 26 years. This selection was necessary to eliminate factors associated with age, at both extremes. Data were usable for 45 experimental and 41 control cases. Raw scores, in terms of number of words read per minute,

are appropriate to compare groups. The median is the midpoint, above and below which 50 percent of the cases fall; it is not as sensitive as the mean to a few extreme cases at either end. The mode is the score occurring most frequently. In this case, the mode of both groups, on both test occasions, is 0, showing that more members of both groups, on both occasions, have 0 anisometropia than any other score. The location of the median between the mean and mode shows clearly the nature of the asymmetry of the distribution.

TABLE 8

FORM FIELD RESULTS: MEANS AND STANDARD DEVIATIONS OF FORM FIELD ESTIMATES FOR EXPERIMENTAL AND CONTROL GROUPS ON PRE- AND POSTEXAMINATIONS

Variable		Experimental Group		Control Group	
		O.D.	O.S.	O.D.	O.S.
No. of cases		75	74	56	56
Pre-test	Mean	28.89	28.03	32.48	32.79
	S.D.	8.57	8.54	9.29	9.36
Posttest	Mean	38.77	39.19	28.32	28.64
	S.D.	7.56	7.41	7.95	8.23
Difference		9.88	11.16	−4.16	−4.15
t		13.96	13.61	7.95	6.05
p		<0.01	<0.01	<0.01	<0.01

TABLE 9

READING SPEED RESULTS: MEAN AND STANDARD DEVIATION OF T SCORES REPRESENTING DIFFERENCES BETWEEN NUMBER OF WORDS READ PER MINUTE AT PRE- AND POSTTESTS BY EXPERIMENTAL AND CONTROL GROUPS (SUBJECTS BETWEEN 11 AND 26 YEARS ONLY)

Variable	Experimental Group		Control Group
No. of cases	45		41
Mean (difference)	57.13		42.59
Standard deviation (difference)	6.60		5.81
t (of mean difference)		11.74	
p		<0.01	

were used to obtain differences between pre- and posttests. These differences were transformed to T scores, with mean of 50 and standard deviation of 10. The results are shown in Table 9.

These data indicate a significant difference in favor of the trained subjects (experimental group) in improvement of reading speed.

RELATION OF INITIAL STATUS TO CHANGE

Inasmuch as several writers have stated that only low degrees of myopia can be reduced by training, the relation of initial visual measurement to final and change measures were computed for visual acuity, refractive error, and form field. The correlations are shown in Table 10. For visual acuity, correlations were computed for O.D. and O.S.; without correction, only, since these will be representative of the visual acuity findings.

The pre- and postmeasures are all highly correlated, those for refractive error being the highest. These correlations indicate a high degree of relationship between the early and late measures for the same individuals, reflecting the reliability of these functions. The relative magnitude of the correlations for refraction, visual acuity, and form field is roughly inversely proportional to the amount of change reported earlier and shown in Tables 4, 5, 6, and 8. The generally lower correlations for the experimental group for visual acuity and form field may further be interpreted to indicate that the rank order within this group is changed more as a result of training, whereas the rank order within the control group, which received no training, is less disturbed by the mere passage of time.

In contrast to the pre-post correlations, those between initial measures and differences (reflecting change from pre- to postmeasures) are much lower. All of the cor-

TABLE 10

CORRELATIONS BETWEEN PRE- AND POSTMEASURES OF VISUAL ACUITY, REFRACTION AND FORM FIELD AND BETWEEN PRE- AND CHANGE (POST-PRE) MEASURES

Variable		Experimental Group				Control Group			
		r(pre-post)	No.	r(pre-change)	No.	r(pre-post)	No.	r(pre-change)	No.
Visual Acuity	O.D.sc	0.87†	80	0.32†	80	0.97†	60	0.04	60
	O.S.sc	0.82†	80	−0.27*	80	0.95†	60	−0.09	60
	O.U.sc	0.87†	76			0.96†	59		
	O.D.cc	0.72†	75			0.87†	58		
	O.S.cc	0.73†	75			0.80†	58		
	O.U.cc	0.68†	71			0.80†	57		
Refraction[1]	O.D.	0.97†	70	−0.27*	70	0.97†	58	0.30*	58
	O.S.	0.96†	71	−0.31*	71	0.96†	58	0.15	58
Form field[2]	O.D.	0.72†	75	−0.51†	75	0.88†	56	−0.49†	52
	O.S.	0.74†	74	−0.54†	74	0.84†	56	−0.33*	51

* $p<0.05$.
† $p<0.01$.
[1] Refraction, based on retinoscopy under cycloplegic, converted to spherical equivalent.
[2] Form field, in degrees, based on average of 90- and 180-degree radii on perimetry chart.

relations for the experimental group are significant at at least the five-percent level and all are negative, indicating, contrary to expressed opinions cited above, that those with the greatest deviation below normal tended to improve the most. This tendency is greatest for form field and about equal for visual acuity and refraction. For the control group, the corresponding visual acuity correlations are insignificant, which is understandable in view of the extremely high correlations between pre- and postmeasures. One of the two correlations for refractive error, for the control group, is marginally significant but the signs of both coefficients are positive, suggesting that the trend in direction of deterioration is for the more myopic cases to be most progressive. The control group correlation coefficients for form field are comparable with, but only slightly lower than, those for the experimental group. The meaning of these relationships is not clear.

Relations among visual acuity, refractive error, and form field measures

Up to this time the various measures used in this study have been considered separately. It is of interest to examine the degree of relationship among them and this has been analyzed for the three of greatest interest.

Table 11 shows the intercorrelations of pre- and postmeasures, separately, for visual acuity (without correction), refractive error, and form field. These correlations were computed for O.D. only, as representative of the relationships involved.

The correlation between visual acuity and refractive error is 0.58 and 0.70, respectively, for experimental and control groups on pretest and 0.66 and 0.68 on posttest. These relationships are significant but nevertheless leave almost 60 percent of the common variance between the two measures unexplained.* Hence, there are many possible factors accounting for each which are independent of the other and it is possible for changes to occur in visual acuity as a result of such factors (for example, visual control and perceptual habits) without reference to concomitant changes in refractive error.

The correlations of visual acuity and form field are all significant, but lower than those just examined. As in the previous correlations, these are also higher, but not significantly so, for the control group. These correlations again show a significant positive relationship but leave about 75 percent of the common variance unexplained. Hence it is

* The percent of common variation among two variables is estimated by the square of the correlation coefficient. Thus, if $r = 0.60$, 36 percent of the variation in each is attributed to the same factor, whereas if $r = 0.90$, 81 percent of the variation is related to a common factor. The presumption of common factors related to their variation increases as their correlation increases.

TABLE 11

Intercorrelations of visual acuity (without correction) refractive error and form field, O.D. only, for pre- and postmeasures

Correlation Coefficient	Experimental Group r	Experimental Group No.	Control Group r	Control Group No.
Pre-visual acuity w. refractive error	0.58†	72	0.70†	59
Pre-visual acuity w. form field	0.32†	74	0.49†	53
Pre-refractive error w. form field	0.05	68	0.36†	55
Post-visual acuity w. refractive error	0.66†	73	0.68†	56
Post-visual acuity w. form field	0.26*	74	0.44†	49
Post-refractive error w. form field	0.04	70	0.33*	49

* $p < 0.05$.
† $p < 0.01$.

possible for either to be affected by training without reference to the other.

The correlations between refractive error and form field are close to zero for the experimental group and low, positive, and significant for the control group. The differences between the two groups are not easy to understand and must be regarded an anomalous, as in the case of the form field correlations in Table 10.

Generality of change

In view of the uniform results with reference to visual acuity, refractive error, and form field, the experimental group generally improving and the control group generally showing deterioration, it is of much interest to examine whether such changes are generalized, that is, associated with each other. This has been done by computing the intercorrelations among the change measures, separately for each group, as shown in Table 12.

Examination of Table 12 indicates that the correlations for both groups cluster about zero; none is significant. It may, therefore, be concluded that change on any variable among the three included is independent of change on the others.

Discussion of results

In one respect the results of this study constitute the most favorable demonstration of visual training effects known to us. This is the sweeping generality and consistency of the changes in visual functions obtained in both the experimental and control groups. In this section the magnitude and practical significance of these results will be evaluated and some hypotheses will be advanced to account for them.

TABLE 12

Intercorrelations of change measures (post-pre) for visual acuity (without correction), refractive error and form field, O.D. only

Correlation Coefficient	Experimental Group		Control Group	
	r	N	r	N
Visual acuity w. refractive error	−0.03	70	−0.13	56
Visual acuity w. form field	0.06	74	0.26	49
Refractive error w. form field	0.04	66	−0.01	48

The most striking changes occurred in visual acuity. The changes in form field and in reading speed were highly significant, but not as great. The changes in refractive error were significant, but small. Although the changes in these functions were unrelated, as seen in their intercorrelations, they occurred uniformly in the direction of improvement for the trained experimental group and uniformly in the direction of poorer visual performance for the untrained control group.

The visual acuity results are summarized in terms of original scores and transformations (table 5). The average improvement in uncorrected visual acuity of the trained subjects was 64 percent for O.D. and O.S. and 56 percent for O.U. This is equivalent to an improvement of four lines on the AMA test chart. The corresponding changes for this group, with correction, are less, as might be expected. They were 19 percent for O.D. and O.S. and 11 percent for O.U., which is equivalent to a gain of one line or less on the AMA test chart. Ninety-three percent of this group showed some improvement.

The average loss in uncorrected visual acuity of the control subjects was 27 percent for O.D. and O.S. and 26 percent for O.U., which is equivalent to about two to three lines on the AMA test chart. The average loss in visual acuity with correction is slightly less, 24 percent for O.D. and O.S. and for O.U., or about one to two lines. Ninety-eight percent of the control cases had some loss in visual acuity from initial to final examination.

The average increase in size of form field for the experimental group was 37 percent while the average decrease for the control group was 13 percent. Ninety-six percent of

the experimental cases had an increase, while 88 percent of the control cases had a decrease.

The corresponding changes in refractive error were as follows. The improvement (that is, reduction) for the experimental group was nine percent (0.22D.), while the average loss (that is, increase) for the control group was 12 percent (0.29D.). Sixty-nine percent of the experimental cases had some reduction of refractive error, while 79 percent of the control cases had some increase.

If the losses in the control group were progressive, they would be severely handicapped without a change in their lenses at the end of a year and probably would require much stronger myopic correction within a few years. It is unlikely that loss of visual functions would be progressive so generally or that debilitative processes would be so rapid. This observation raises questions concerning the meaning of the control group results.

Two hypotheses must be considered in this connection. The first is that the ophthalmologists who performed the examinations were aware of the status of subjects they examined as members of either experimental or control group and that their examination results reflected constant errors as an inadvertent effect of such knowledge. Such an explanation might readily account for the results on refractive error, since one quarter of a diopter, which is the average change of each group, is within the range of error in accurate determination of refractive error by retinoscopy. It might also account for the visual acuity and form field results of the control group and part of the results of the experimental group, although the magnitude of differences obtained in these functions is greater than might reasonably be explained as simple errors of measurement.

The ophthalmologists who performed the examinations were interviewed with reference to this possibility. They were not specifically aware of the status of the subjects examined, although they did record their findings on the record sheets, which did identify the subjects. These doctors were not convinced advocates of the training technique, but professed open-minded scientific attitudes in co-operating in the research. Hence, they were not biased in favor of a particular result. Nevertheless, it is possible that some of the bias in the data could be accounted for on the basis of inadvertent knowledge. However, even if the amount of change in the control group was taken as a liberal estimate of constant error, the residual improvement in the experimental group in visual acuity, form field, and reading speed would be substantial. Following this reasoning, the refractive differences would be cancelled out and the net visual acuity improvement due to training would be 30 percent (two lines), while the net increase in size of form field would be 20 percent.

The second hypothesis is derived from the differences in observed motivation of the two groups. It may be said that the co-operative subjects, who strongly desired to take the training and report regularly and promptly, tended to be placed in the experimental group, while the unwilling, or less co-operative, subjects, who were unable to or uninterested in keeping their training appointments, tended to gravitate into the control group. Since the most substantial results, namely those for visual acuity, form field, and reading speed, involve functions which depend in large part on learning, it is reasonable to expect that the more highly motivated subjects would improve as a result of consistent practice and effort.

The motivation hypothesis does not explain the losses of the control group. However, taking this together with the first hypothesis, of inadvertent measurement bias due to knowledge of subjects' status, a meaningful explanation of the findings may be proposed. If this is accepted, the importance of motivation in visual training is highlighted and this may further explain the differences between the favorable results of this experi-

ment and the less favorable results obtained by other investigators cited earlier. In such research an objective measure or rating of motivation is indicated, as demonstrated by the foregoing discussion.

In view of the foregoing discussion, the mean net improvement obtained in the trained group, after subtracting the mean decrease in visual performance of the control group, as a correction for constant errors due to knowledge of subject's status and motivational differences, is accepted as a valid estimate of the effects of tachistoscopic training in this study. This has been found to be 30 percent in visual acuity and 20 percent in size of form field. With this correction, the changes in refractive error are cancelled out.

These results have important implications. It is reasonable that refractive error, measured by retinoscopy under cycloplegia, should be resistant to change purely as a result of visual practice. This measure of refractive error is primarily a function of the structural, anatomic, optical properties of the eye, plus errors of measurement. Visual acuity and form field, on the other hand, involve learned habits of seeing, such as associating various perceived cues with objects, of searching and fixating, in addition to the optical qualities of the eye. Hence, it is also reasonable to find improvement in these functions by practice, even in myopic patients, especially since they were so highly motivated.

It is not possible to assign relative importance to the structural and behavioral components of these functions; however, one might also raise a question concerning the limits of improvement which could be expected through the type of tachistoscopic visual training employed in the present study. The training schedule followed covered a period of 10 weeks. It is probably most appropriate to note that the limits of improvement were not tested in this study and that this is a problem for further research. Additional training, periodic testing during training, and certain variations in the training curriculum should be investigated further. Nothing can be said, on the basis of the data presented, about the duration or permanence of the gains obtained. This, too, is an important problem for further investigation.

Summary and Conclusions

This experiment was conducted to test the effects of a program of tachistoscopic visual training, developed by Renshaw[47] on several visual functions in myopic subjects. Two groups of structural myopic subjects, uncomplicated by an ocular pathologic, alteration, were used. The experimental group consisted of 80 subjects who were judged to be highly motivated to participate in and benefit by the training program. The control group included 60 subjects whose motivation was judged to be poor, if not somewhat negative, despite the fact that they, too, were volunteers. The two groups were well matched in age, sex, education, socio-economic status, and initial visual measurements.

Both groups received pre-examinations by ophthalmologists, which included retinoscopy under cycloplegia, visual form fields, visual acuity, retinal rivalry rate, motility studies, and routine ophthalmologic examination of the eyes. The experimental group then received three tachistoscopic training sessions per week for 10 weeks, while the control group received no training, but followed their normal routines during this period. At the completion of the training course, the two groups received final examinations identical to the initial examinations. The effects of training were determined for the experimental group in terms of the differences between initial and final measurements and these were compared with the corresponding differences for the control group, which received no training.

The raw results indicated a general trend toward improvement in visual acuity, size of visual form field, refractive error, and reading speed in the experimental group, and a similar trend toward deterioration of these

functions in the control group. The following specific results were obtained:

1. Ninety-three percent of the experimental group improved in visual acuity without correction. The average increase was equivalent to four lines on the AMA test chart, of 56 to 64 percent, from 20/128 to 20/79 (O.D., sc), 20/121 to 20/75 (O.S., sc), and 20/98 to 20/63 (O.U., sc). These changes were significant beyond the 0.01 level. Similarly significant, but smaller increases of 19 percent (O.D. and O.S.) and 11 percent (O.U.) were found for visual acuity with correction.

2. Ninety-eight percent of the control group incurred a loss of visual acuity which averages 26 to 27 percent, from 20/110 to 20/157 (O.D., sc), 20/120 to 20/157 (O.S., sc), and 20/97 to 20/131 (O.U., sc). The losses in visual acuity, with correction, were of the same magnitude. All differences were significant beyond the 0.01 level.

3. The average increase in visual form field was 37 percent for the experimental group, while the average decrease for the control group was 13 percent. Ninety-six percent of the experimental cases increased while 88 percent of the control cases decreased. The differences were both significant beyond the 0.01 level.

4. The corresponding changes in refractive error were an average reduction of 0.22D. for the experimental group (nine percent improvement) and an increase of 0.29D. for the control group (13 percent loss). Sixty-nine percent of the experimental cases had some reduction of refractive error, while 79 percent of the control cases had some increase.

5. The mean amount of anisometropia in both groups ranged from 0.41D. (experimental group) to 0.45D. (control group). No significant changes were observed in either group.

6. The experimental group read a significantly greater number of words per minute after training than the control group after a comparable period of time.

7. Correlations between initial measures and change scores tended to be negative for the experimental group, suggesting that the subjects with most impairment increased the most. These correlations were significant, but low: -0.27 to -0.32 for visual acuity, -0.27 to -0.31 for refractive error, and -0.51 to -0.54 for form field. This result is contrary to opinions expressed in the ophthalmologic literature.

8. Changes in visual acuity, refractive error, and visual form field, in both groups, were uncorrelated with each other. Initial measures of these variables were positively correlated, but the magnitude of the correlations was such that a large proportion of the common variance was unaccounted for, suggesting that substantial changes in any of these variables might occur without reference to the others.

The losses observed in the control group were analyzed and interpreted as accountable to a large extent as constant errors resulting in part from knowledge of the subjects' status on the part of the ophthalmologists who performed the examinations and in part from the poor motivation of the subjects. In order to obtain a conservative appraisal of the positive effects of training on the highly motivated experimental group, the average loss of the control group might be taken as a liberal estimate of error and subtracted from the average gains of the experimental group. This would give the following results: (1) no change in refractive error; (2) 30 percent (two lines) improvement in visual acuity (without correction); and (3) 20 percent increase in size of visual form field.

This estimate of the effects of the specific course of training in the present experiment is believed to be conservative. The motivation of the trained subjects was, however, a noteworthy condition distinguishing this study from others reported in the ophthalmologic literature.

Investigators who may do further research in this field may well profit by the experience

of this study and take additional precaution to assure that controls recognized in the experimental design are actually achieved in the data. It is suggested that further research should explore the limits of training improvement which might be derived from additional or somewhat varied training curricula and should include follow-up studies to investigate the permanence of improvements obtained. The need for including objective measures or ratings of motivation, in evaluating results, is obvious. Such measures would be a major addition to research methodology in this field.

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Discussion

Dr. Henry A. Imus (Bethesda, Maryland): It is both a pleasure and an honor to be invited to discuss this most interesting and important paper on the question concerning the effects of visual training on myopic patients.

As the authors have indicated, this is a controversial subject, and many points of view have been expressed rather strongly in the literature. In my own opinion, this report greatly clarifies the situation.

In clinical work it is difficult to establish and maintain controls, and it is most difficult to make sure that all of the subjects obey the rules and return for follow-up examinations and tests. I think that the authors and their associates are to be congratulated upon the degree of success they have achieved in this matter.

Since one third of the control group were subjects dropped from the experimental group for unsatisfactory attendance, this may bias results somewhat. It would be interesting to know how the visual acuity and refractive errors of these 20 subjects compared with the experimental group. Also, if any further research is conducted on this problem, neither the examiner nor the subject should know whether the individual being examined belongs to the control or experimental group. This so-called "double-blind" technique is proving to be a very valuable tool in clinical research.

It is difficult to understand the greater variability in refractive error and visual acuity in the experimental group as compared with the control group, as shown in Table 2. If these two groups were strictly comparable, one might expect the variability in these measures to be equal.

It is difficult to understand, also, the deterioration of the control group in both refractive error and visual acuity. The difference in time between test and retest for the two groups does not offer a logical explanation.

The improvement in apparent visual acuity as a result of the training given to the experimental group is statistically significant. That this is due to learning to interpret blurred images rather than to marked changes in refractive error is well established. The increase or decrease of the latter of approximately one-quarter diopter is not very important, albeit statistically significant, when the probable error of measurement is one-eighth diopter. In this connection, it would have been most valuable to have determined the test-retest reliability of the examiners themselves.

If possible, it would be most interesting to repeat this experiment, using the controls as the experi-

mental group and the trained subjects as the control group. The pretest in such an experiment would show the relative permanence of the differences demonstrated in this experiment.

Again, may I say, this has been a difficult job very well done under the circumstances of dealing with clinical patients of lower than average educational achievement, some of whom were poorly motivated.

DR. T. E. SANDERS (Saint Louis): I should like to ask Dr. Berens a question, because we were co-authors on a similar project a number of years ago.

We found that practically any myopic patient who had an error of over two diopters received no benefit from the training. It was only the patient with a low degree of myopia who showed improvement. We felt the higher myopes showed little improvement and, therefore, we felt that this was one of the big objections to the method. In other words, the degree of myopia had much to do with our results. I wonder if Dr. Berens found that also.

DR. LOUIS J. GIRARD (closing): The investigators wish to thank Dr. Imus for his kind discussion. We are certainly cognizant of the many variable and uncontrollable factors that were present in this investigation.

We were unable to explain the variability in refraction and the visual acuity in the experimental group. We also were unable to explain the degree of deterioration of the control group in such a short period of time.

The investigators agree with Dr. Lancaster that the improvement in visual acuity which results from training is probably an improvement in perception, probably in the interpretation of blurred images.

It would have been very valuable to have tested the test-retest reliability of the examiners as suggested by Dr. Imus. This was not checked. To answer Dr. Sanders, it was found in our study that the higher degrees of myopia showed the greatest change, which was exactly the opposite of the Saint Louis study.

THE HUMAN OPTIC PAPILLA*

A DEMONSTRATION OF NEW ANATOMIC AND PATHOLOGIC FINDINGS

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INTRODUCTION

The optic papilla represents a very important part of the human eye. It is composed of neurites, glia, connective tissue, and blood vessels, and has an extremely complicated architecture. Pathologic changes in human eye diseases involve very often the optic papilla. It must be emphasized here that the optic nerve and papilla are parts of the central nervous system and not of a true peripheral nerve.

In the present contribution it is intended to demonstrate new anatomic and pathologic findings which may explain some of the common clinical observations following involvement of the optic nervehead in eye diseases. This study is a continuation of the two earlier demonstrations on "the astroglia of the human retina and other glial elements of the retina under normal and pathologic conditions"[1] and "reactions of the elements of retina and optic nerve in common morbid entities of the human eye"[2] which were read before this association in 1955 and 1956.

MATERIAL AND METHOD

The human eyes used in this study were obtained either after surgical enucleation or at post mortem. All eyes were fixed in formalin or bromformalin. The silver carbonate methods of del Rio Hortega were used to stain frozen sections of the optic nerves of the eyes. These methods make it possible to stain selectively the neurites and the glial cells as well as the blood vessels and the connective tissue structures of the optic

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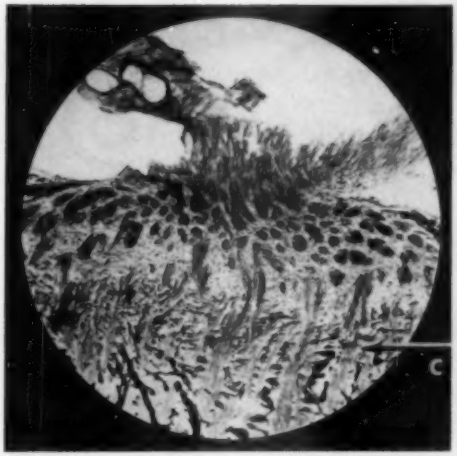

Fig. 1 (Wolter). Longitudinal section of optic nervehead of normal human eye. The nerve-fiber bundles are stained. The lamina cribrosa is slightly visible (c). The astroglia is not impregnated. (Hortega method, photomicrograph.)

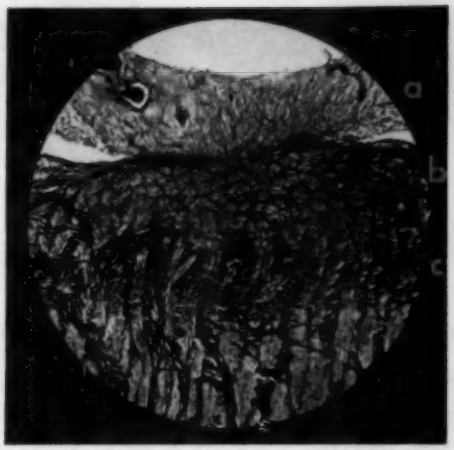

Fig. 2 (Wolter). Glia stain of longitudinal section of normal human optic nervehead. (a) Nerve fiber layer of the retina. (b) Basketlike anterior portion of the lamina cribrosa. (c) Lamina cribrosa. (Hortega method, photomicrograph.)

papilla and their pathologic changes. The potentialities and the technique of these methods were described in detail by Scharenberg and Zeman.[3]

All illustrations contained herein are unretouched photomicrographs.

Histologic description

The optic papilla virtually represents a round sievelike structure at the posterior pole of the globe. Its architecture provides for a well-protected outlet of all neurites of the nerve-fiber layer of the retina which form the optic nerve. There is as little weakening of the dense outer layer of the eyeball as possible.

The supporting structures of the papilla consist of a posterior and anterior portion (comp. fig. 2). The posterior portion is formed by a sievelike, intertwined structure of connective-tissue fibers and is well known as the lamina cribrosa of the optic nerve. This structure is actually a part of the sclera. The anterior portion of the optic papilla resembles a shallow, caplike wicker basket (figs. 2 and 3) and is composed of special retinal astroglia.[4] This anterior glial part of the human optic papilla has been widely ignored in the literature.

All neurites of the nerve-fiber layer of the retina undergo in the papilla a 90-degree flexion from the retina into the optic nerve (fig. 1). It is the function of the caplike wicker basket mentioned above to protect and support these neurites at this vulnerable point of flexion. This basket is composed of a special type of astroglia with long processes (glial fibers) and small cell bodies. These cells are known as "spider cells" (fig. 7) (comp. Marchesani[5]). They are of neuroectodermal origin. These cells form dense networks of glial fibers around the bundles

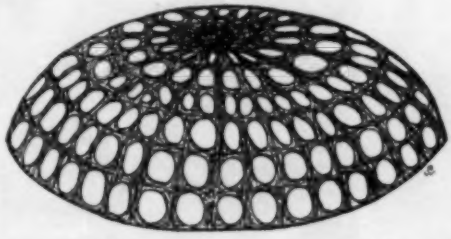

Fig. 3 (Wolter). Drawing reconstruction of the caplike formation of spider cells of the anterior portion of the lamina cribrosa.

of neurites and around single nerve fibers with most of their long processes. But they also send many processes to the capillaries which are contained within this basket and form dense sheaths around them. This glial sheathing of the capillaries results in a functional separation between the mesodermal tissues of the blood vessels and the neurites.

The glial basket of "spider cells" is closely connected to the lamina cribrosa with its base (fig. 2). Many holes in the anterior surface of the caplike apparatus contain the bundles of neurites and its structures lead these bundles in a gradual flexion toward the perforations in the lamina cribrosa. This glial basket is connected to the lamina cribrosa with its posterior surface (base). It is also connected at its margin with Bruch's membrane of the choroid, and with the ring-shaped spore of the sclera at the margin of the disc. The arrow in Figure 4 indicates the area of this connection between the "spider cells" of the anterior portion of the papilla, Bruch's membrane, and the scleral spore.

Obviously, the caplike anterior glial portion of the optic papilla is an important supporting, protective, and nutritive organ of the optic nerve fibers which traverse it. It has mostly a round anterior surface (fig. 3). This surface, however, may normally have a central impression if there is a physiologic excavation of the optic disc.

The sievelike structure of the lamina cribrosa is normally of a very regular and delicate pattern (fig. 5). Intertwined connective tissue fibers which are found to be a direct continuation of the scleral connective tissue form many round holes for the bundles of neurites. These fibers are also directly connected with the wall of the central blood vessels. The perforations of the lamina cribrosa, however, contain not only the nerve fibers but also their accompanying system of astroglia which separates the connective tissue fibers from the neurites (fig. 8). This system represents the supporting and nutritive structure of the single nerve fibers. The whole apparatus of the lamina cribrosa therefore resembles a coarser network of connective-tissue fibers which contains another more delicate network of astroglia in its spaces (fig. 8).

No hypertrophy or active proliferation of the elements of the anterior glial basket of the optic papilla was observed under patho-

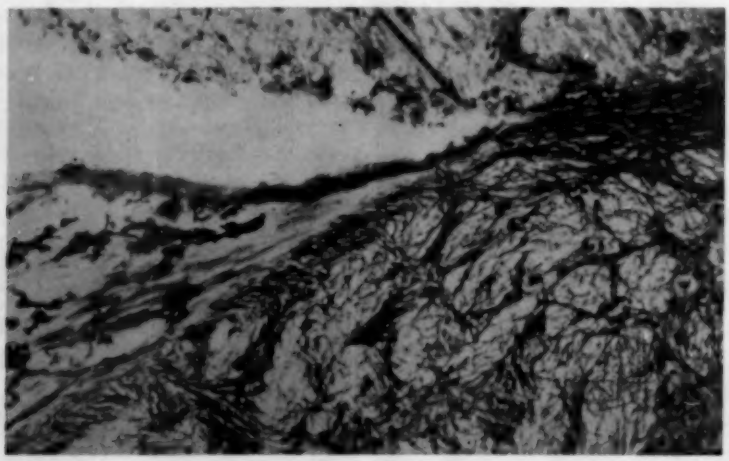

Fig. 4 (Wolter). High-power view of a glia stained section of the optic nervehead shows the connection of Bruch's membrane, the scleral spore at the disc margin, and the surface of the basket of spider cells (arrow). (Hortega stain, photomicrograph.)

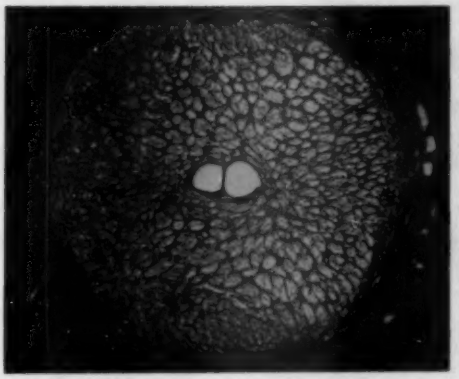

Fig. 5 (Wolter). Cross section of the normal human optic nervehead through the area of the lamina cribrosa. The delicate architecture of the lamina cribrosa and its anchorage in the surrounding sclera and in the walls of the central vessels are visible. The dark-stained surrounding represents the dense scleral connective tissue. (Hortega method, photomicrograph.)

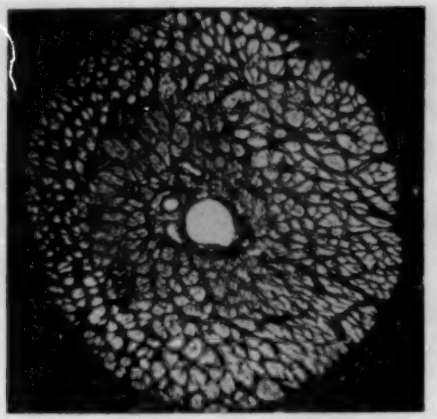

Fig. 6 (Wolter). Hypertrophy of the lamina cribrosa in a case of optic nerve atrophy following advanced retinitis pigmentosa. The connective tissue fibers of the lamina cribrosa are much coarser, and the central artery is much smaller than normal. (Hortega method, photomicrograph.)

logic conditions. This basket, however, shows advanced atrophy in all pathologic situations which result in degeneration of the optic nerve fibers. A virtually complete destruction of this glial basket can often be observed in cases of advanced glaucoma.

Hypertrophy of the glial and connective tissue structures of the lamina cribrosa occur in cases of primary and secondary optic nerve atrophy. Newly formed connective

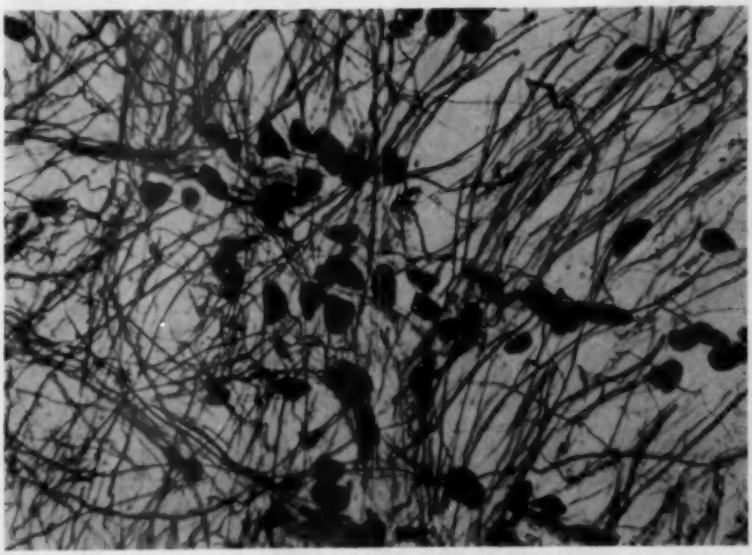

Fig. 7 (Wolter). High-power view of the spider cells of the anterior portion of a normal human optic papilla. (Hortega method, photomicrograph.)

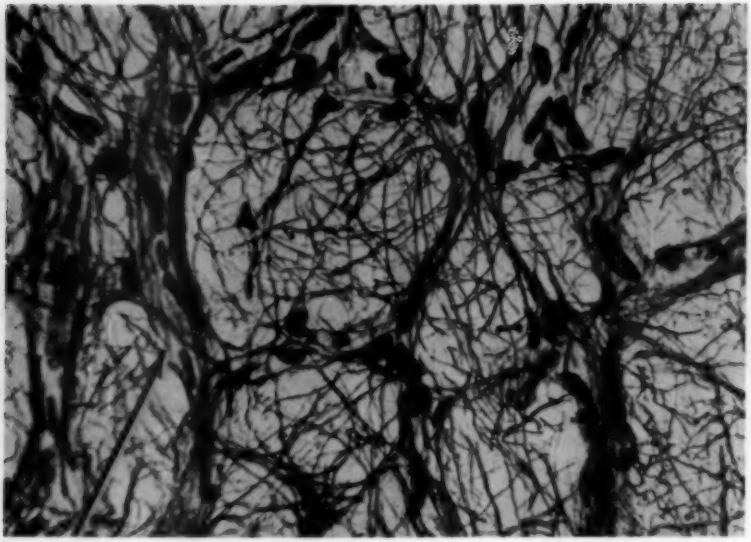

Fig. 8 (Wolter). High-power view of glia-stained section of the lamina cribrosa of a normal human eye. The connective tissue structures (arrow) which contain the blood vessels are only slightly impregnated. The glia fibers in the spaces of the coarser network of connective tissue fibers represent a delicate network. (Hortega stain, photomicrograph.)

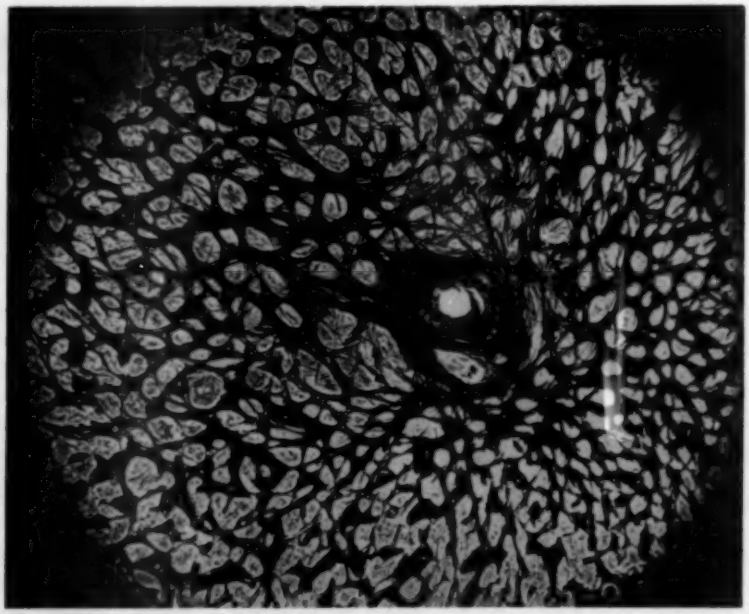

Fig. 9 (Wolter). Extreme hypertrophy of the connective tissue structures of the lamina cribrosa in a case of advanced secondary glaucoma. (Cross section of optic nervehead, Hortega method, photomicrograph.)

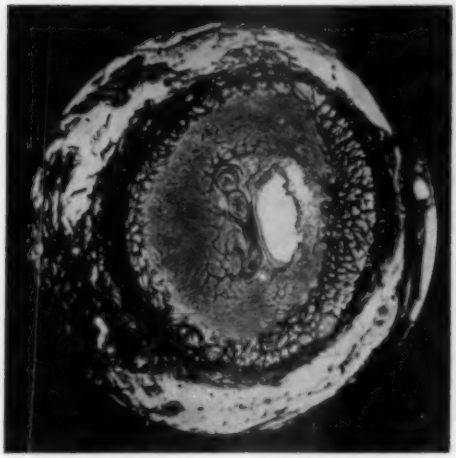

Fig. 10 (Wolter). Parallel section to Figure 9 at a more anterior level. The lamina cribrosa takes part in a deep glaucomatous excavation. The anterior basket of spider cells is compressed into a dense shallow layer of glial cells. The nerve-fiber layer has virtually disappeared. (Cross section, Hortega method, photomicrograph.)

tissue fibers and hypertrophic astroglia then replace the degenerative nerve fibers.

Figure 5 shows a flat section through the lamina cribrosa of a normal eye. Figure 6 for comparison represents a similar section of an eye with optic nerve atrophy following advanced retinitis pigmentosa. The hypertrophy of the supporting structures is clearly visible; there is extensive atrophy of the nerves and the central artery is very small.

Figure 9 exhibits extreme hypertrophy of the lamina cribrosa as seen in a case of advanced secondary glaucoma in a 70-year-old man. Figure 10 represents a parallel section to Figure 9 at a more anterior level. This latter section shows that the lamina cribrosa in this case takes part in the formation of a deep glaucomatous excavation. The spaces of the lamina cribrosa contain virtually no nerves and are filled with the scarlike fibers of hypertrophic astroglia (fig. 11). The anterior basket of spider cells is compressed into a dense, shallow layer at the inside of glial cells on the glaucomatous cup (fig. 10).

Fig. 11 (Wolter). Shows a high-power view of the hypertrophic structures of the lamina cribrosa (a) and the scarlike astroglia in their spaces (b). (Cross section, Hortega method, photomicrograph.)

Posteriorly the lamina cribrosa has no sharp limits and is closely connected with the connective tissue structures of the pial septa of the optic nerve. The latter contain the blood vessels. Longitudinal sections of

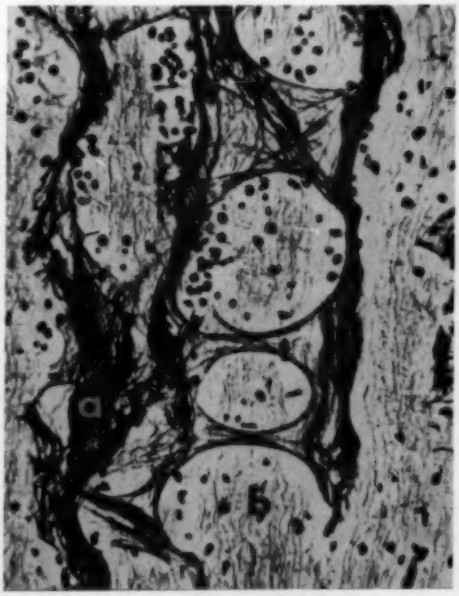

Fig. 12 (Wolter). Longitudinal section of normal human optic nervehead directly posterior to the lamina cribrosa. The connective tissue structures are black stained (a). The nerve fibers are only slightly stained but can be recognized (b). (Hortega method, photomicrograph.)

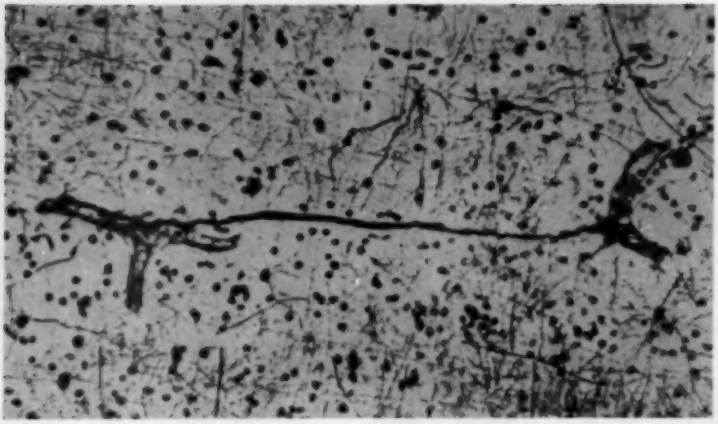

Fig. 13 (Wolter). Shows a similar bridgelike connection of small blood vessels by connective tissue fibers in the white matter of the human brain. (Hortega method, photomicrograph.)

the optic nerve give a good impression of the complicated architecture of these septa (fig. 12).

Most of the meningeal fibers of the optic nervehead run in a longitudinal direction parallel to the blood vessels. Archlike fiber structures connect and reinforce these coarse formations at an angle of about 90 degrees. It is interesting to note that very similar bridges of connective tissue fibers as seen between the blood vessels of the optic nerve (fig. 14) are also seen between blood vessels of the white matter of the brain (fig. 13).

All connective tissue structures of the

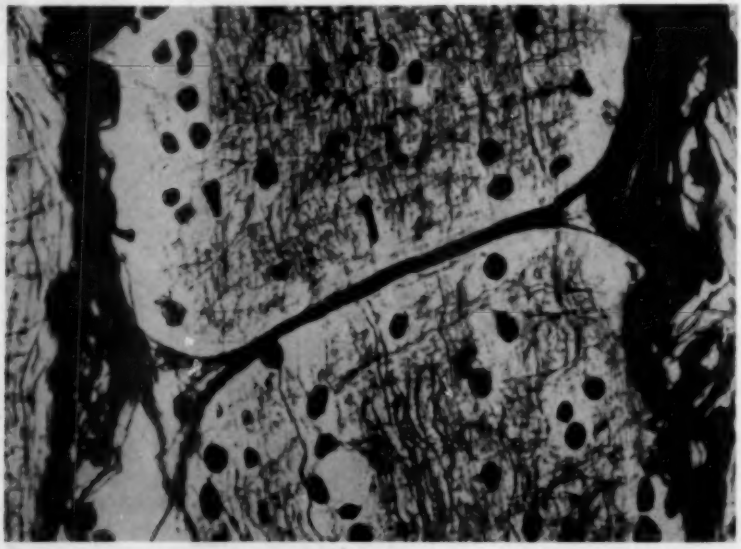

Fig. 14 (Wolter). Longitudinal section of normal human optic nervehead which shows the bridgelike connection of two small blood vessels of the leptomeninges by a strand of connective tissue fibers. (Hortega method, photomicrograph.)

lamina cribrosa and the optic nerve are firmly anchored in the sclera of the globe or the dural sheath of the optic nerve (fig. 15). The loose network of bizarre connective tissue fibers of the arachnoid layer and pial layer of the optic nerve represent the connection between the dense structures of the optic nerve (fig. 15).

Knowledge of the architecture of the optic papilla is important for understanding of its pathology in papilledema. The existence of the loosely arranged spider cells and the lack of Mueller's radial fibers in the anterior portion of the papilla explain the fact that a localized swelling and protruding of the disc can occur (comp. Henderson[6]). The surrounding retina possesses Mueller fibers which connect the outer and the inner limiting membrane. These fibers do only allow for a limited swelling of the retina. An extensive swelling of the retina occurs in papilledema usually much later in the clinical course and is explained by the destruction of the Mueller fibers at the disc margin. The

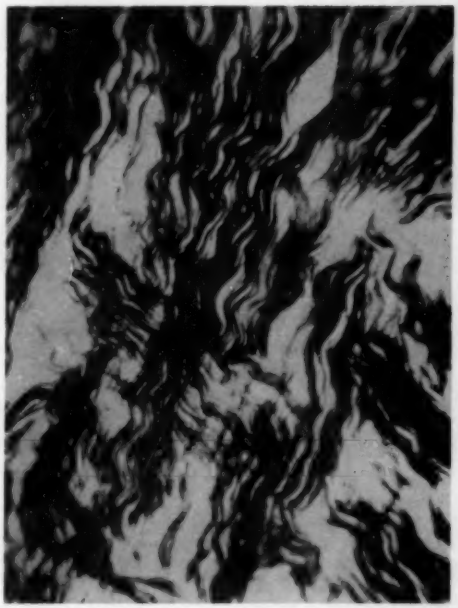

Fig. 15 (Wolter). The connective tissue fibers of the dural sheaths of the normal human optic nerve. (Hortega method, photomicrograph.)

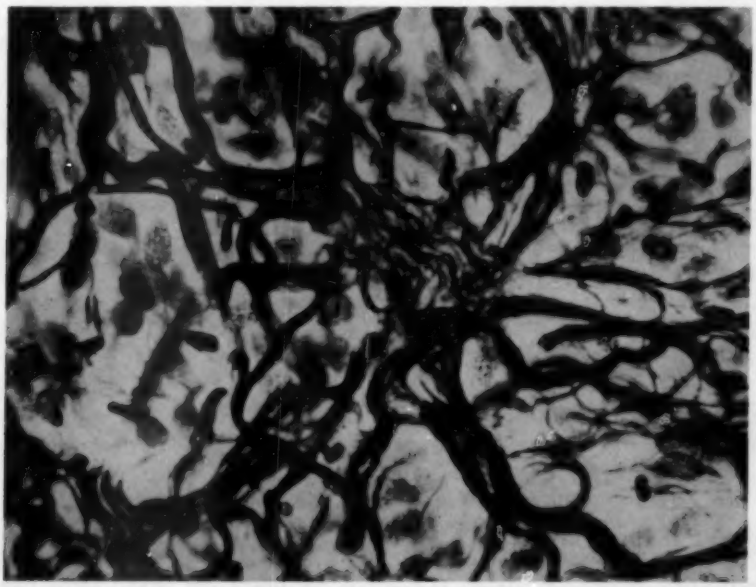

Fig. 16 (Wolter). Bizarre connective tissue fibers of the arachnoid layer of the normal human optic nerve. The nuclei of arachnoidal cells are slightly stained. (Hortega method, photomicrograph.)

Fig. 17 (Wolter). Low-power view of the swollen retina at the nasal disc margin of a case of advanced bilateral papilledema. Arrow I indicates the area from which the high-power view Figure 20 was taken. Arrow II indicates the area from which Figures 18 and 19 were taken. (Hortega method, photomicrograph.)

elasticity of the glial basket of the papilla probably allows for a certain amount of swelling without irreversible damage to the neurites. The structures of lamina cribrosa, however, are not so elastic and seem to be the direct cause for the destruction and interruption of the nerve fibers in late stages of papilledema.

Figure 17 represents a low-power view of the swollen retina at the nasal disc margin of a case of advanced bilateral papilledema. The picture is taken from a slide of the left eye of a 56-year-old man who died with elevated intercranial pressure and very extensive cerebral edema following an infarct in the left hemisphere and a diagnostic arteriography. The nerve-fiber layer in this disc is swollen and protruding. It occupies about four times its normal thickness. There also is destruction of all retinal structures—including the radial fibers of Mueller—in an area of retina surrounding the disc. The latter destruction of retina tissue at the disc margin and its replacement by swollen degenerative nerve fibers is accepted as the cause for the enlarged blindspot of the visual field in advanced papilledema.

It is very interesting to study the neurites of the nerve-fiber layer at the disc in this case. Especially nasally most of these neurites are interrupted. They exhibit bizarre terminal swellings (Cajal) at the distal ends of the proximal segments of their axones. The bulblike formations are clearly visible at the margin of the buttonlike protruding papilledema in Figures 17 and 18. More advanced destruction, swelling, and fragmentation of the neurites can be seen right on the disc (fig. 20).

The bulblike terminal swellings here observed are the same as those seen in the cotton-wool patches of the retina (Wolter,[7] Wolter, Goldsmith and Phillips[8]). It is known from general pathology that such terminal swellings always develop in the central and peripheral nervous system following the interruption of nerve fibers at the site of their injury. We believe that an accumulation of terminal swelling as seen on the disc of this case of papilledema contributes to the whitish color of the papilledema as observed ophthalmoscopically.[9] However, there is also interfibrillar exudate which is also known to result in a whitish discoloration (as seen, for example, in retinal edema). The most severe nerve-fiber damage is found in the nasal part of the posterior portion of this papilla where the neurites traverse the lamina cribrosa (fig. 20).

Histologic studies of old cases of papilledema with secondary optic-nerve atrophy show that most of the neurites of the nerve-fiber layer, the optic papilla, and the optic nerve disappear after some time. Secondary retrograde degeneration of the interrupted nerve-fiber stumps is considered the reason for this fact.

It is interesting to note that one practically never sees a case of histologically complete optic-nerve atrophy. There are always some nerve fibers left. Even three human optic nerves, the eyes of which were enucleated 11 and 16 years ago, were still found to con-

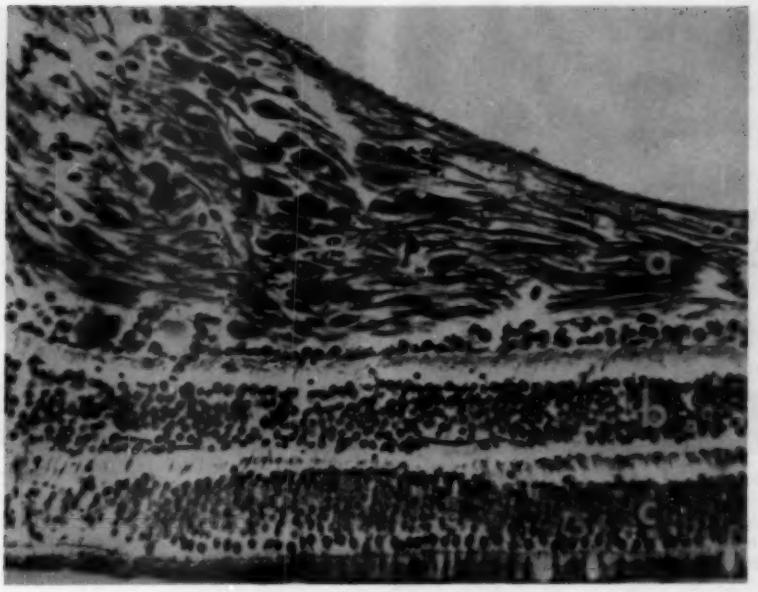

Fig. 18 (Wolter). High-power view of the retina at the margin of the papilledema. Numerous terminal swellings of interrupted nerve fibers are visible in the nerve fiber layer. (a) Nerve fiber layer. (b) Inner nuclear layer. (c) Outer nuclear layer. (Hortega method, photomicrograph.)

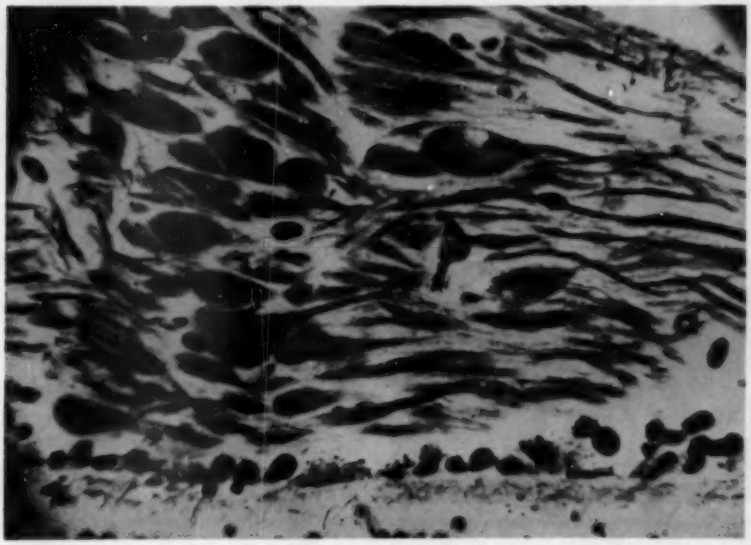

Fig. 19 (Wolter). High-power view of the terminal swellings of interrupted nerve fibers at the nasal disc margin of the nerve fiber layer (a). All these swellings are directed toward the optic disc. (Hortega method, photomicrograph.)

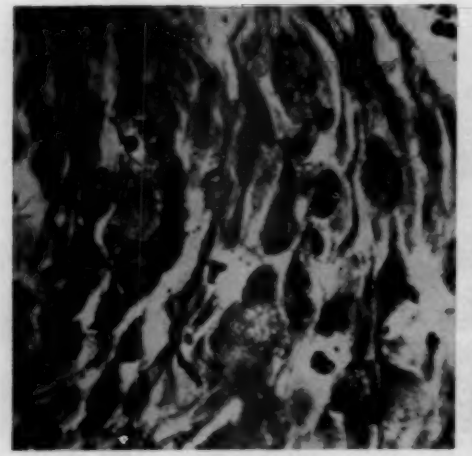

Fig. 20 (Wolter). Advanced destruction, swelling, and fragmentation of neurites on the optic disc of the case of papilledema. (Area of arrow No. II in Figure 17). (Hortega method, photomicrograph.)

tain numerous nerve fibers (Wolter and Liss[10]). This finding is considered evidence for the existence of centrifugal nerve fibers in the human optic nerve which originate in the brain and not in the retina.

Often one is also surprised to find many

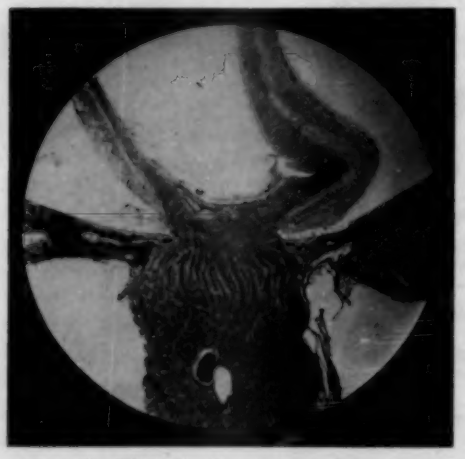

Fig. 21 (Wolter). Low-power view of optic nerve, papilla, and retina of an advanced case of Coats' disease. Many nerve-fiber bundles can be seen within the optic papilla. (Hortega method, photomicrograph.)

Fig. 22 (Wolter). Low-power view of a melanoma of the optic nervehead. (Hortega method, photomicrograph.)

well-preserved nerve-fiber bundles in the optic papilla in cases with complete blindness and very advanced degeneration of the retina. Figure 21 shows the nerve bundles of the optic papilla in such a case. This was a later stage of Coats' disease with total blindness, retinal detachment, degeneration, and development of extensive connective tissue scars in the retroretinal space. Figure 22 represents another example of an advanced involvement of an optic disc in which the optic nerve still contained many nerve fibers. This is a case of a melanoma of the optic nervehead.* However, the optic papilla may sometimes show complete atrophy and then no nerve fibers are found within it. This is most often seen in advanced primary or secondary glaucoma and in late stages of papilledema.

Drusen of the optic nervehead are a very impressive clinical and histologic finding. The drusen represent histologically polymorphous lamellae masses of hyaline substance. I do not believe that they represent a specific pathologic formation. But they

* This specimen was sent to us by Dr. L. E. Zimmerman of the Armed Forces Institute of Pathology in Washington, D.C.

may develop under different conditions which result in an accumulation of necrotic substance on the disc accompanied by poor blood supply. This generalization does not apply to the occurrence of bilateral drusen of the nervehead in normal eyes with normal visual acuity and visual fields (comp. Walsh,[16] p. 418). Figure 23 represents a typical large drusen of the optic nervehead of the left eye of a 20-year-old woman, with blindness, old retinal detachment, and chronic uveitis. As Dr. Heath[2] pointed out in the discussion of my talk before this association in 1956, hyalinization of necrotic tissue obviously is a rather unspecific process which may develop under many different situations. It is interesting that similar conditions which lead in one case to the formation of drusen of the optic nervehead may result in the so-called cavernous atrophy in another case. This latter condition is caused by liquefication of the same necrotic substance which

Fig. 23 (Wolter). Typical large drusen of the optic nervehead of a case with blindness, old retinal detachment, and chronic uveitis. In some areas of the drusen lamellar formations of hyaline are visible. There is complete degeneration of all nerve fibers within the optic papilla. (Hortega method, photomicrograph.)

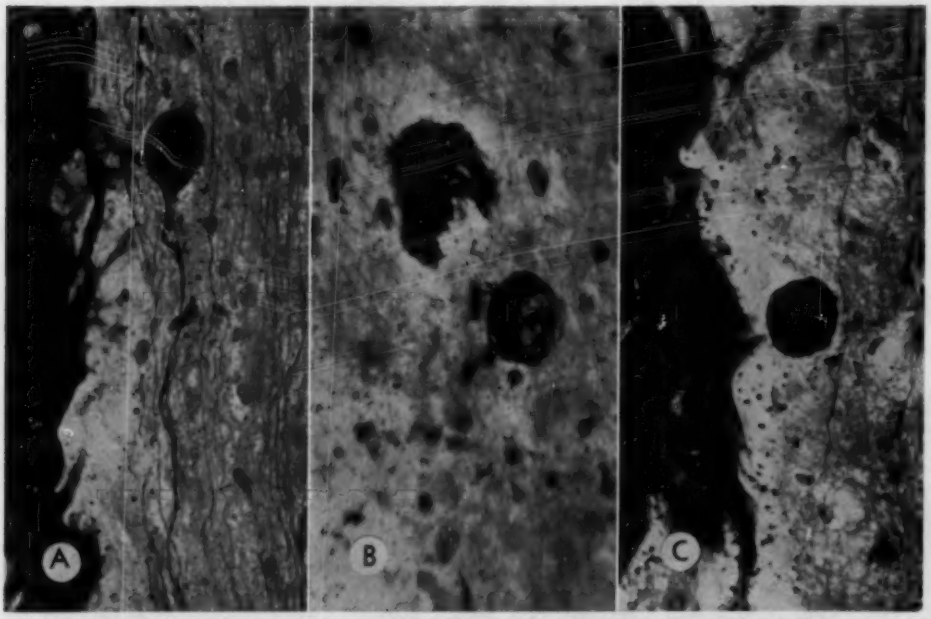

Fig. 24 (Wolter). (A) Interrupted nerve fiber of the optic nervehead which exhibits bulblike terminal swellings at its distal end. (B) A lipoid body left over after retrograde degeneration of the nerve stump of an interrupted nerve fiber in the nervehead. (C) Hyalinization of a round lipoid body as seen in (B). All three pictures are taken from slides of a case with primary optic-nerve atrophy following intracranial tumor compression. (Hortega method, photomicrograph.)

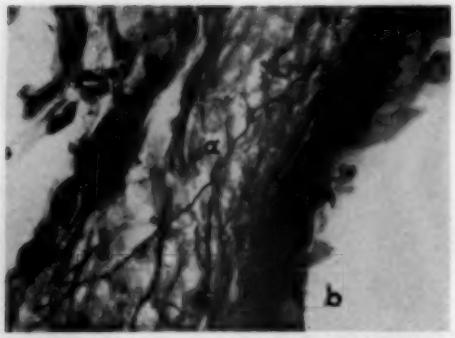

Fig. 25 (Wolter). A centrifugal nerve fiber (a) of the human retina terminating in the wall of retinal blood vessels (b). (Hortega method, photomicrograph.)

becomes hyalinized when drusen develop (comp. Zimmerman[11]).

Small round hyaline corpuscles are a very common finding in the nerve-fiber bundles of the optic papilla under normal and pathologic conditions. They are very similar to those found in the nerve-fiber layer of the retina and in the optic nerve itself. It was reported last year before this association[2] that such bodies may develop under certain conditions from degenerating astroglia as well as from nerve fibers. Figure 24 shows different stages of the development of such hyaline bodies from interrupted neurites in the optic nervehead of a 75-year-old woman with optic nerve atrophy following intracranial tumor compression. Figure 24a shows the terminal swellings of an interrupted nerve fiber. Figure 24b shows a lipoid body which is left from such a terminal nerve fiber swelling after further retrograde degeneration which resulted in the destruction of the more proximal part of the neurites. Figure 24c represents a hyaline body which obviously developed by hyalinization of a lipoid body as seen in Figure 24b. All transitional stages between the end-bulbs of nerve-fiber stumps and the final hyaline bodies can be seen in this case.

It has already been mentioned that there is evidence that some of the nerve fibers of the optic nerve, the papilla, and the nerve-fiber layer of the retina originate in the brain and are, therefore, centrifugal in nature.

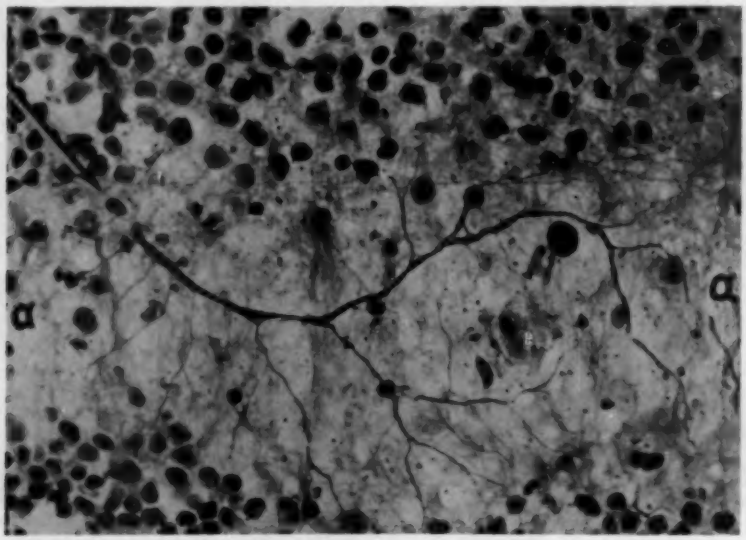

Fig. 26 (Wolter). This slide represents a tangential section through the adventitia of a blood vessel of the human retina (a-a). The terminal branching of a centrifugal (vasomotor) nerve fiber can be seen on the vessel wall. (Hortega method, photomicrograph.)

It is possible to show histologically that some of these nerve fibers supply the blood vessels of the optic nerve and the retina (Liss and Wolter,[13] Wolter[12]). Figures 25 and 26 show as an example the terminations of such centrifugal vasomotor nerve fibers in the wall of large blood vessels in the human retina. It must be mentioned here that the vascular nerves of this type represent only one of two nerve types found in the wall of the blood vessels of the human retina and optic nerve. The second nerve fiber type is represented by a very delicate system of networklike perivascular fibers.[12, 13] The existence of these two different vascular nerves in the human retina and optic nerve is considered histologic evidence for a double autonomic innervation of their blood vessels (sympathetic-parasympathetic?).

The astroglia of the optic papilla gradually changes its character as one proceeds posteriorly into the optic nerve. The astroglia of the latter is similar to that of the white matter of the brain.[14] Figure 27a represents a typical example of an astrocyte of the normal human optic nerve. The drawing reconstruction (fig. 27a) shows that these cells form a dense glial sheathing around the blood vessels with their "sucker-footlike" processes. Their other processes (glial fibers) surround the neurites with a dense supporting and nutritive network. These astrocytes have a small cell body and may normally have one or more round nuclei (fig. 28). For comparison, astrocytes of the white matter of the human brain with their footlike endings on the blood vessels and the glial fibers are demonstrated in Figure 29.

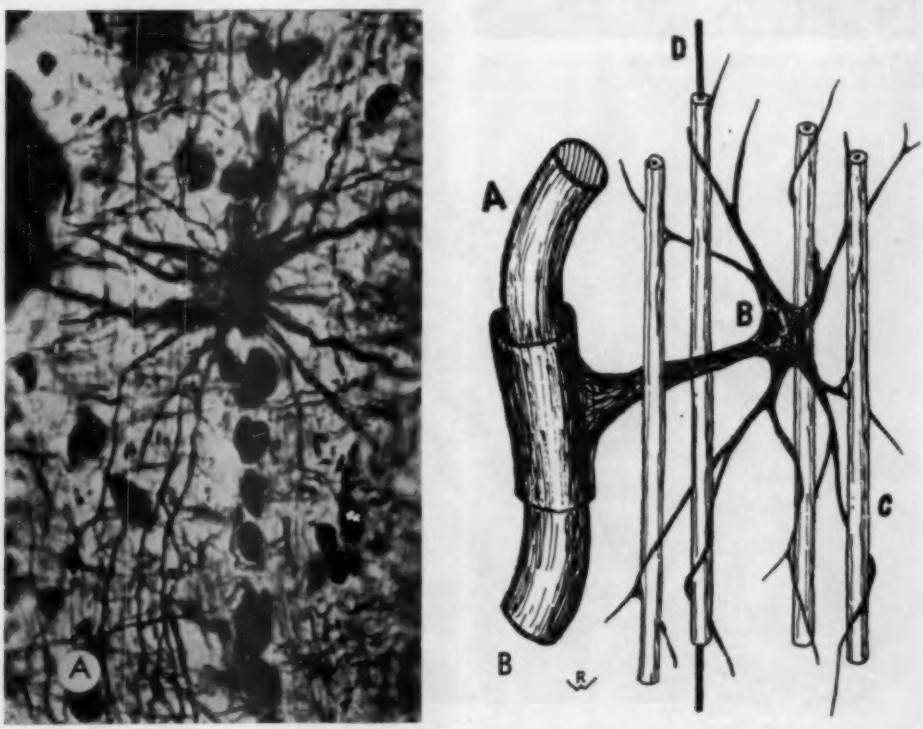

Fig. 27 (Wolter). (A) Astrocytes of the normal human optic nerve. (Hortega method, photomicrograph.) (B) Drawing reconstruction of an astrocyte of the human optic nerve (b) and its relationship to the blood vessels (a) and the neurites (d).

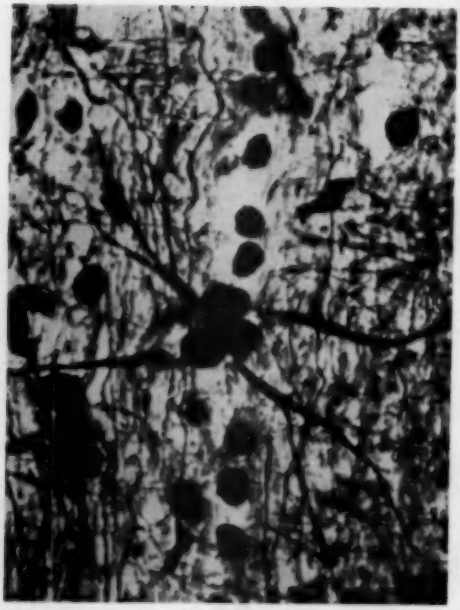

Fig. 28 (Wolter). Astrocyte of the normal human optic nerve which exhibits three cellular nuclei. (Hortega method, photomicrograph.)

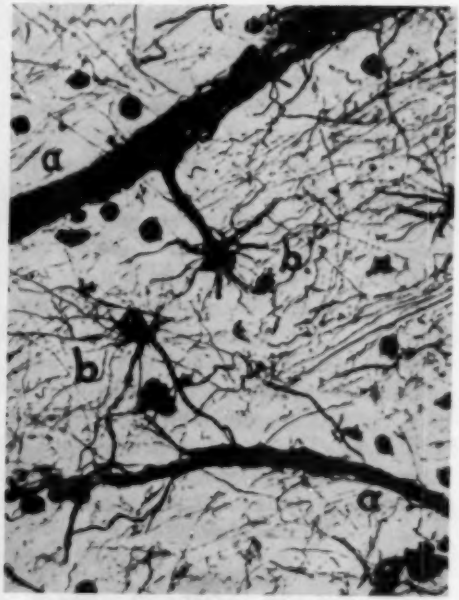

Fig. 29 (Wolter). Shows a section of the white matter of the human brain with its blood vessels (a) and its astrocytes (b). The astrocytes send sucker-footlike processes to the blood vessel wall. (Hortega method, photomicrograph.)

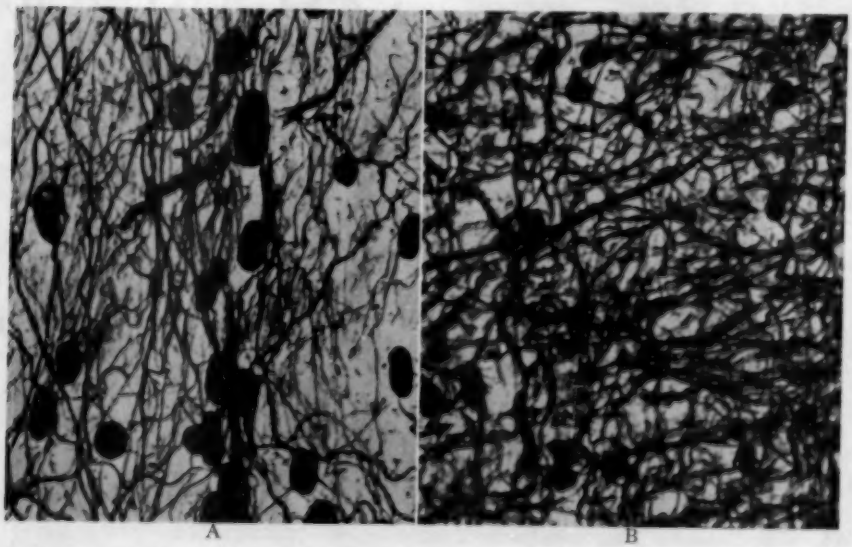

Fig. 30 (Wolter). (A) Hypertrophy and proliferation of the astroglia in a case of primary optic nerve atrophy. (B) Gliosis of the optic nerve in retinitis pigmentosa. (Hortega method, photomicrographs.)

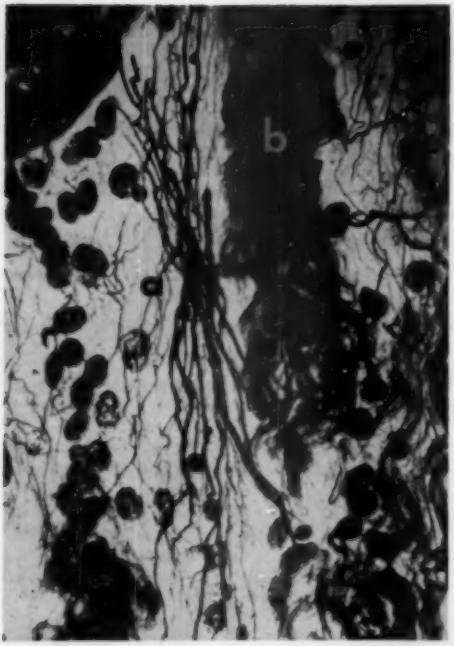

Fig. 31 (Wolter). Advanced scarification and gliosis of the optic nerve stump of a patient whose eye was enucleated 11 years ago. (a) Hypertrophic astrocytes. (b) Hypertrophy of the septal connective tissue. (Hortega method, photomicrograph.)

Hypertrophy and proliferation of the astroglia is very often seen in human eye pathology following atrophy of the nerve fibers. The astroglia then fills the space of broken-down neurites and virtually forms a glial scar. Figure 30 demonstrates two typical slides with such a gliosis of the optic nerve in primary optic nerve atrophy (fig. 30a) and in retinitis pigmentosa (fig. 30b). Figure 31 represents a slide of an optic nerve stump, the eye of which was enucleated 11 years before examination. In this case there is not only gliosis but also hypertrophy of the septal connective tissue.

We have not yet been able to achieve a

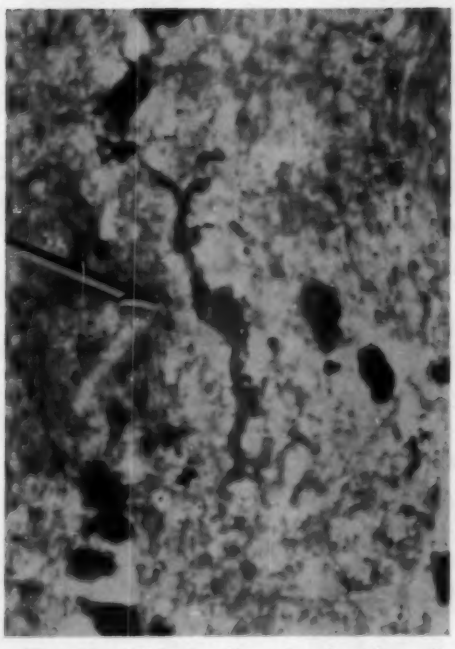

Fig. 32 (Wolter). Normal microglia cells (arrow) of the human optic nerve. (Hortega method, photomicrograph.)

Fig. 33 (Wolter). Accumulation of gitter cells (microglia after phagocytosis) between the remnants of broken-down neurites in a case of neuromyelitis optica. (Hortega method, photomicrograph.)

good staining of the oligodendroglia of the human optic nerve under normal conditions. However, there is indirect evidence for the existence of such cells in the human optic nerve under normal conditions. They correspond to the Schwann cells of true peripheral nerves. Oligodendroblasts and abnormal oligodendrocytes were found in a case of glioma of the optic nerve (Liss and Wolter[15]).

There always is microglia (Hortega glia) in the optic nerve. They normally represent small cells with short processes and round nuclei (fig. 32). The microglia are generally known as the phagocytes of the central nervous system and represent the reticuloendothelial element of retina, papilla, and optic nerve. The microglia is of mesodermal origin. It is their function to phagocytize the remnants of destroyed neurites of the optic nerve under pathologic conditions. The active microglia represents large lipoid-laden cells with a small round nucleus. They are then known as "gitter cells" or granular compound corpuscles. Figure 33 shows as an example an accumulation of such gitter cells between the remnants of broken-down neurites in a case of neuromyelitis optica.

This study gives just a few new aspects of the anatomy and pathology of the optic papilla as found with techniques which stain selectively the nerves and the glia. The new findings with these methods are very numerous and often difficult to interpret. Therefore, the present demonstration should be considered only as a beginning and cannot at all be complete. It is our hope, however, to extend the knowledge of the anatomy and pathology of the nervous and glial structures of the human eye within the years to come and to introduce the study of these elements as part of histologic routine in eye pathology.

University Hospital.

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Discussion

Dr. Parker Heath (Sullivan Harbor, Maine): This paper is concerned with the morphology and some of the pathologic reactions of the optic papilla and the optic nerve. The findings are related to previous studies by the author, using the same techniques. The latter employ brom-formol and formol-fixed material, frozen sections, stained by Hortega's silver carbonate.

The method discriminates the neurites, certain of the neurons, blood vessels, and connective tissue. Unfortunately for us, this method has been little used in recent years on ocular tissue, except the revealing work of Dr. Wolter and those associated with him.

His study of the optic papilla depicts a complex of two parts—an anterior cap or basket of retinal astroglia perforated by neurites individually and in bundles, the latter insulated by a feltlike mesh of long glial processes. Interconnections of glial dendrites pass to capillaries and to single and bundles of neurites. The cell bodies of the astroglia form the nuclear columns and stain out small and round, with processes so numerous that the common term for them is "spider" cells.

The anterior part of the nervehead or the neuro-ectodermal cap is attached to Bruch's membrane, to the choroid, and posteriorly to the lamina cribrosa, as well as to the wall of the scleral passageway. It seems to me that the inner or vitreal boundary is indicated in some of his illustrations as a condensation of fibers, but this is not described by the author. The anterior cap has acquired added morphologic significance from Dr. Wolter's study.

The lamina cribrosa he describes conventionally from the findings of his method as a perforated sievelike continuation of the sclera, composed of connective tissue. Within its meshes are found the nerve fibers and bundles. The neurites are accompanied and insulated by a columnar system of astroglia.

The anterior neuro-ectodermal cap and the posterior fibrous scleral sieve jointly create a seal or gasket between intraneural and intraocular regions. Both transmit centrifugal and centripetal nerves and insulate them and, in addition, convey their blood supply, as well as that for the retina. Pial and arachnoid fibers connect with the lamina cribrosa. The pia carries capillaries and larger vessels. Sometimes the blood vessels are connected by a bridge of connective tissue, as in the brain.

The principal pathologic reaction of the astroglial condensation composing the anterior cap of the papilla is degeneration. The neurites passing through and the cap suffer the same fate. Degeneration of one is accompanied by degeneration of the other. The actual changes are characteristic of neural tissue, as swelling and fragmentation, degeneration and atrophy.

The connective tissue and accompanying glia of the lamina cribrosa, on the other hand, may react to injury by proliferations whose effects are to replace the bulk of the lost nerve fibers.

The author portrays especially well the sequence of events on the nervehead with degenerating neurites. Bulblike terminal swellings similar to those seen in the retina as cotton-wool spots, are common. They are to be found more on the mesial side of the disc where the fiber layer is more compact and increase the hazard from pressure.

He also has observed that following advanced retinal or optic nerve atrophy the loss of neurites is not complete. This has supported other older observations which suggest the existence of both centripetal and centrifugal fibers.

The author's work shows beautifully two types of terminations from the neurites which enter the nerve from the brain and apply to blood vessels of the optic nerve and the retina: one is an aborizing form, the other a network. Especially well shown by the technique are the form and distribution of the astrocytes in the optic nerve. These cells may serve as intermediaries between the blood supply and the neurites.

The method so far has not been successful in depicting the oligodendrocytes; we know that they are present normally and in great excess with gliomas of the optic nerve. The mesodermal phagocytosing microglia are well shown, especially when fat-ladened. They are then known as "gitter" cells or granular compound cells. Only samplings of pathologic states are reported, that is, late stages of primary optic-nerve atrophy, advanced retinal atrophy, drusen, and cytoid bodies.

We can't have everything, but the addition of full descriptions of the blood supply in health and disease of this region would markedly round out our knowledge. The author's work is a great credit to him; the method is well established, as his brilliant photomicrographs testify.

CONTINUING STUDIES ON THE ASSOCIATION OF ADENOVIRUS TYPE 8 WITH EPIDEMIC KERATOCONJUNCTIVITIS*

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Epidemic keratoconjunctivitis is a serious eye disease of well-defined clinical characteristics which occurs both sporadically and in epidemics all over the world. After an incubation period of seven to 10 days, the typical case develops an acute follicular conjunctivitis and preauricular adenopathy. About 10 days later, round, subepithelial corneal opacities appear, which may persist up to two years and seriously interfere with vision. Varying proportions of patients during epidemics develop corneal involvement. Since only the corneal lesions are entirely pathognomonic for the disease, certain individuals having only conjunctival involvement cannot be diagnosed with absolute certainty. This is often the case in children,[1] and makes the diagnosis of sporadic or slightly atypical cases of the disease difficult, until a laboratory test for etiologic diagnosis can be established.

The etiology of epidemic keratoconjunctivitis is not established at the present time. Claims have been made for the role played by many different viruses, but most of them have not, and cannot, be substantiated.[2] Most available textbooks list a virus (Sanders) as the etiologic agent. Currently available strains of that virus resemble St. Louis encephalitis virus. The position of that agent and of others for which claims have been made has been well evaluated recently by Cheever.[3] It is clear that the available strains of this virus bear no serologic relationship to the disease as it has existed since 1951. In the present paper we wish to present evidence that adenovirus type 8 (APC 8) has been regularly associated with epidemic keratoconjunctivitis since 1951 in several parts of the world, and that this agent is capable of causing the disease.

Material and Methods

Patients

Some of the patients included in this study were sporadic cases seen in the eye clinic of the University of California Medical Center. Much material was obtained through the cooperation of ophthalmologists and virologists in many areas, including Dr. H. L. Ormsby, Toronto; Dr. M. D. Pearlman, Chicago; Dr. Irving Leopold, Philadelphia; Dr. H. Konig and Dr. R. Witmer, Switzerland; Dr. G. Bietti, Italy; Dr. R. Biehling, Dr. H. Hofmann, and Dr. H. Moritsch, Austria; Dr. M. Inouye and Dr. Y. Mitsui, Japan; Dr. O. Vivell, Germany; and Dr. R. G. Sommerville and Dr. D. Tyrrell, Great Britain. We are deeply indebted to these investigators for their help. Epidemic keratoconjunctivitis has existed in epidemic or endemic form in all of these areas since 1951. The patients ranged in age from one to 75 years, and were classified as typical epidemic keratoconjunctivitis, atypical or probable epidemic keratoconjunctivitis, or eye disease clinically not epidemic keratoconjunctivitis.

Virus isolations

Conjunctival or corneal scrapings were taken, using a sterile platinum spatula, from lesions of patients with active conjunctivitis or keratitis. The material was suspended in tubes containing maintenance medium (10-percent chick serum in mixture 199 with antibiotics), and inoculated immediately into

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HeLa cell tissue cultures. The cultures were incubated in a stationary position at 36°C. for several weeks, with occasional changes of maintenance medium, and were observed at frequent intervals for cytopathogenic effects. If no such effects appeared, at least one blind passage was made before the material was discarded as negative.

Sera

Whenever possible, paired serum specimens were obtained from each patient, the first immediately after onset, the second after an interval of a few weeks. In some instances, serial specimens were obtained over a period of two months. Single convalescent sera were obtained from patients who had previously been diagnosed as having typical epidemic keratoconjunctivitis. Comparable sera, single or paired, were obtained from control populations in the same geographic areas, selected from similar age groups. All sera were stored at −20°C. until used.

Serologic tests

The technique for the neutralization test has been described.[4,5] Sera were inactivated at 56°C. for 30 minutes and diluted in mixture 199. Undiluted virus and diluted serum were mixed in equal volume, incubated at room temperature for one hour, and inoculated into tissue cultures, usually HeLa cells. Cytopathogenic effects were recorded as 0 to 4+ in the conventional manner. Neutralizing antibodies were considered present only if there was a difference of at least 3+ between control (normal rabbit serum) and experimental tubes for two consecutive days. Complement-fixation tests were performed as described elsewhere.[4]

Volunteer inoculations

The procedure has been outlined elsewhere.[6] Individuals showing no clinical signs and no history of past infection with epidemic keratoconjunctivitis were selected. In some instances these patients were kept in isolation for 10 days before inoculation. Serum specimens were obtained before inoculation, at the time of onset of any disease, and at intervals thereafter. The inoculations were made either by scarification of the anesthetized conjunctiva or by simple instillation of centrifuged supernatant fluid from HeLa cell cultures of adenovirus type 8. Material from uninoculated tissue cultures was inoculated into the other eye simultaneously.

Results

1. Isolation of viruses

To date at least nine strains of adenovirus type 8 have been isolated in tissue culture, as shown in Table 1. The prototype strain

TABLE 1

Strains of adenovirus type 8 isolated to date

Patient	Age	Geographic Area	Clinical Diagnosis	Keratitis Present	Infectivity*
Trim	50	California	Typical EKC	+	$10^{-1.0}$–$10^{-1.5}$
Koy	27	Japan	Probable EKC	−	$10^{-2.0}$–$10^{-2.5}$
Iji	70	Japan	Typical EKC	+	$10^{-2.0}$–$10^{-2.5}$
Nag	13	Japan	Probable EKC	−	$10^{-2.0}$
Ish	58	Japan	Typical EKC	+	$10^{-1.0}$–$10^{-1.5}$
Kam	1	Japan	Probable EKC	−	$10^{-2.0}$
? (Dr. Chang)	? (Child)	Saudi-Arabia	Unidentified conjunctivitis	?	?
Seg (Dr. Tanaka)	45	Japan	Probable EKC	+	?
Law (Dr. Tyrrell)	? (Adult)	Scotland	Typical EKC	+	$10^{-2.0}$–$10^{-2.5}$

* Highest dilution of virus (tissue culture fluid) giving cytopathogenic effects in HeLa cells.

(Trim) was recovered in California,[4,5] six strains in Japan,[7,8] and one strain in Scotland.[9,10] These eight strains were derived from patients with clinically unequivocal epidemic keratoconjunctivitis. The ninth strain was isolated by Murray et al.[11] in Saudi-Arabia from the eye of a child suffering from an unidentified conjunctivitis. It is remarkable that adenovirus type 8 has to date been isolated from the eye only, and not from other tissues which have readily yielded other types of adenoviruses.

Adenovirus type 8 differs in several biologic characteristics from other members of that group. Cytopathogenic effects in tissue culture in original isolations are usually delayed for two to three weeks. The infective titer for HeLa cells is always low (rarely exceeding 10^{-2}), but the amount of antigenic material in tissue culture is as great as with other types of adenovirus. In any form the virus is extraordinarily stable, withstanding shipment either as lyophilized or fresh tissue culture, or as epithelial shreds from conjunctival scrapings. Like other adenoviruses, the agent withstands repeated freezing and thawing without loss of titer. In contrast to other types, disruption of tissue culture cells fails to increase the infective titer.

While adenovirus type 8 has been isolated up to the 12th day after onset of the conjunctivitis, it is probably recovered most readily in the first five days.

2. Antibodies to adenovirus type 8 developing during the illness

Paired or serial serum specimens taken at intervals of two weeks or more were available from 40 patients. Dr. R. G. Sommerville[9] kindly made available information on four additional individuals. In 36 of these 44 persons there was a fourfold or greater rise in neutralizing antibody to adenovirus type 8. Of the remaining eight, six had a titer of 1:10 or greater, and possibly might have shown a fourfold rise had additional sera been obtained at longer intervals. Heterotypic rises to other types of adenovirus were infrequent and always less than fourfold. The optimal time interval between the acute and convalescent serum specimens is not well established. A few patients already had high titers of neutralizing antibodies (1:20 or greater) when first seen, during the second week of their disease. A majority of patients, however, did not develop such titers until three weeks or more after onset of the eye symptoms. Table 2 shows the relationship between time of serum collection and demonstration of a fourfold or greater rise in antibody titer.

It has been pointed out in earlier publications[5,12] that the titer of neutralizing antibodies in adenovirus type 8 infections may decline rapidly after the acute infection. The above data in Table 2 suggests that a positive serologic diagnosis will be most likely if not two but three sera are examined: An acute phase serum collected during the first week of the illness, a second specimen obtained about three weeks after onset, and a third specimen collected six to eight weeks after onset. Such a pattern will permit the detection of both early and late antibody titers.

In the majority of sera examined by us, the peak titer did not exceed 1:80, and in many patients it remained at 1:40 for some weeks before declining. Perhaps this relatively low concentration of neutralizing antibodies in adults is due to the limitation of the infection to the eye, and the relative absence

TABLE 2

Relationship between time of serum collection and antibody titer

Time Second Specimen* Collected (wk. after onset)	Number of Serum Pairs Showing Rise in Neutralizing Antibody Titer between First and Second Specimen	
	4-fold or greater	Less than 4-fold
2	9	5
3–4	15	1
5–8	17	7
17–52	7	4

* First serum specimen was collected during the first week after onset in these serum pairs.

of systemic symptoms. In small children living in endemic areas, epidemic keratoconjunctivitis apparently occurs as a systemic illness with fever, diarrhea, vomiting, pharyngitis, and otitis, with a pseudomembranous conjunctivitis but no keratitis.[1,13] The few paired sera that have been examined have shown significant rises in antibody titer to adenovirus type 8. In one such child the peak titer was definitely in excess of 1:160 during the third week after onset.

The complement-fixation test does not appear to be a satisfactory serologic method for the diagnosis of adenovirus type 8 infection. Since all types of adenoviruses share a common complement-fixing antigen, many persons have antibody titers in their serum. The rise of complement-fixing antibodies does not parallel that of neutralizing antibodies in many instances.[14,15]

3. Prevalence of antibodies to adenovirus type 8

Initial serologic surveys of sera obtained from various parts of the world indicated that patients with epidemic keratoconjunctivitis had a much higher incidence of neutralizing antibodies to adenovirus type 8 than other persons, of similar age distribution, living in the same areas. We have continued to compile such data, and the present totals are listed by country of origin in Table 3. The validity of this type of serologic evidence relating adenovirus type 8 to clinical epidemic keratoconjunctivitis is predicated upon several assumptions:

a. The virus produces clinically manifest infection more often than subclinical infection.

b. Observers in different parts of the world diagnose epidemic keratoconjunctivitis with the same degree of accuracy and are able to separate epidemic keratoconjunctivitis from morphologically similar entities of different etiology.

c. Only a short time elapses between the acute illness and the collection of serum specimens; otherwise, the rapid decline of antibody titer will make serologic diagnosis of adenovirus type 8 infection impossible.

The data shown in Table 3 are reassuring in several respects. Serologic surveys in the United States and Canada[5,16] indicated a very low incidence of neutralizing antibodies to adenovirus type 8, in contrast to the experience with other adenoviruses. Other countries experiencing large outbreaks in the last decade or having a high endemic level of epidemic keratoconjunctivitis show, as expected, a higher (13.3 to 25.0 percent) incidence of such antibodies. Nevertheless, the incidence of antibodies in patients presumably free from clinical epidemic keratoconjunctivitis never approaches that observed in epidemic keratoconjunctivitis.

The ability of different observers to diagnose clinical epidemic keratoconjunctivitis appears to be quite constant in terms of the

TABLE 3

INCIDENCE OF NEUTRALIZING ANTIBODIES TO ADENOVIRUS TYPE 8 VIRUS IN EPIDEMIC KERATOCONJUNCTIVITIS PATIENTS AND CONTROLS

Geographic Location	Epidemic Keratoconjunctivitis			Non-Epidemic Keratoconjunctivitis		
	No. Sera	Neutralizing Antibodies in 1:10 Serum Dilution		No. Sera	Neutralizing Antibodies in 1:10 Serum Dilution	
		No.	%		No.	%
U.S.-Canada	37	35	94.6	106	5	4.7
Switzerland	15	13	86.7	—	—	—
Italy	15	13	86.7	24	1	4.2
Japan	38	33	86.8	20	5	25.0
Austria	42	22	52.4	30	4	13.3
Germany	18	16	88.9	6	1	16.7
Total	165	132	80.0	186	16	8.6

incidence of antibody to adenovirus type 8 occurring in persons given this diagnosis (86.7 to 94.6 percent) in five widely separated geographic areas. Most of the sera in these five areas had been collected within less than one year after onset of the illness. On the other hand, the sera from Austria were collected in 1956 from patients who had epidemic keratoconjunctivitis during the epidemic of 1952-53. This delay may explain the lower incidence figure (52.4 percent) of antibodies to adenovirus type 8 in the Austrian sera.

In spite of the several reservations regarding such a serologic survey, the overall results appear highly significant: There are 80.0 percent antibodies (1:10) in patients with the diagnosis of epidemic keratoconjunctivitis, as compared to 8.6 percent of such antibodies in fairly well-matched controls without clinical epidemic keratoconjunctivitis. The conclusion appears to be warranted that cases of both sporadic and epidemic keratoconjunctivitis occurring since 1951 have been regularly associated with infection by adenovirus type 8, or an antigenically closely related agent.

4. Inoculation of volunteers with adenovirus type 8

Humans have been inoculated with adenovirus type 8 grown in tissue culture by Dr. Y. Mitsui in Kumamoto, Japan, and Dr. G. Bietti in Rome, Italy. These investigators have kindly permitted us to quote their results. Mitsui et al.[6] have reported in detail the experiences in the first five volunteers. In each case, one eye was inoculated by scarification with adenovirus type 8, the other eye with plain tissue culture fluid. Four of the five volunteers developed epidemic keratoconjunctivitis, with typical subepithelial opacities in three of them. The fifth volunteer had antibodies to adenovirus type 8 at the time of inoculation, and remained well. The uninoculated eye became involved in three volunteers six to eight days after the inoculated eye. Adenovirus type 8 was reisolated from lesions in two cases, and the volunteers developed significant antibody titer rises to adenovirus type 8.

More recently Mitsui has "vaccinated" three volunteers by the subacutaneous injection of cell-free fluid from tissue cultures in which adenovirus type 8 had grown, and subsequently challenged them with the same virus instilled into the eye. One of these volunteers (M. M.) responded to vaccination with a neutralizing antibody titer of 1:80, resisted the first ocular challenge, and, following the second instillation of virus into the eye, developed follicular conjunctivitis with adenopathy but no keratitis. Two other volunteers (T. S. and K. F.) developed no neutralizing antibody following vaccination. They responded to ocular challenge with follicular conjunctivitis, adenopathy, and the typical subepithelial opacities of epidemic keratoconjunctivitis, and a striking rise in neutralizing antibody. The opacities lasted for only a few weeks before reabsorption took place. Thus it appeared that the presence of neutralizing antibodies in significant titer (1:20 or greater) acquired through natural infection or through vaccination may prevent the keratitis of epidemic keratoconjunctivitis.

Bietti had occasion to observe some of Mitsui's volunteers and felt that the experimental disease was entirely typical of epidemic keratoconjunctivitis.[17] He subsequently undertook similar volunteer studies in Italy. Initially, eight persons were inoculated by placing into the eye drops of fluid from tissue cultures in which adenovirus type 8 had been grown. None developed manifest disease. Later, six additional persons were exposed by swabbing virus-containing material onto the scarified conjunctiva. All six developed follicular conjunctivitis with preauricular adenopathy, and two showed the typical subepithelial opacities of epidemic keratoconjunctivitis. Regrettably, serologic studies on these Italian volunteers are not available. However, Bietti observed that seven additional persons de-

veloped, "spontaneously," typical epidemic keratoconjunctivitis through contact with the inoculated volunteers.

The volunteer studies available thus far indicate that inoculation of adenovirus type 8 into the eye of a susceptible person can result in clinical disease entirely compatible with typical epidemic keratoconjunctivitis. The role of trauma, virus concentration in the inoculum, and the role of circulating antibodies present at the time of exposure need to be further clarified.

Discussion

Cheever[3] has recently reviewed some of the claims for the isolation of *the* etiologic agent of epidemic keratoconjunctivitis. Elsewhere, we have outlined[12] the minimum criteria which must be met before such claims may be given serious consideration. Among such criteria is the necessity that viruses be exchanged between different investigators for comparison of results, and that exhaustive attempts be made to identify and classify agents isolated in different laboratories. Regrettably, most reports are seriously deficient in these features. Thus a meaningful discussion of claims made by others is not feasible.

The data presented in this and earlier reports[4, 5, 12] strongly suggest that epidemic keratoconjunctivitis, at least since 1951, has been uniformly associated with infection by adenovirus type 8, and that this agent is one capable of producing an illness indistinguishable from typical spontaneous epidemic keratoconjunctivitis. The virologic and serologic studies conducted independently in Japan,[6–8, 18] Scotland,[9] and Germany[19] support this view. However, at this time it is not possible to state that adenovirus type 8 is the *sole* agent capable of producing epidemic keratoconjunctivitis, or indeed that it was the virus responsible for outbreaks prior to 1951. It must be recalled again that, unfortunately, retrospective serologic investigation is not possible, due to the short persistence of antibodies to adenovirus type 8.

It is appropriate to consider some of the difficulties which persist in establishing a single etiologic agent for epidemic keratoconjunctivitis.

1. While typical cases of epidemic keratoconjunctivitis, with fully established subepithelial opacities, present an unequivocal clinical picture, atypical cases occur both sporadically and during epidemics. Although some of these atypical cases fail to develop corneal changes of any kind, others present an atypical keratitis. It is well established[20–22] that certain individuals infected with adenovirus types 3, 7, or 7 prime, and possibly with other unrelated viruses, may develop changes resembling the keratitis of such "borderline" cases of epidemic keratoconjunctivitis.

If these patients are labeled "epidemic keratoconjunctivitis," then it will have to be concluded that epidemic keratoconjunctivitis is not a single etiologic entity, but represents a sequence of eye tissue reactions to a variety of viral agents. If, on the other hand, the opinion prevails that epidemic keratoconjunctival is a single etiologic entity (with adenovirus type 8 a good candidate at the present time), then laboratory evidence of infection with the designated etiologic agent will be necessary to establish proof of the diagnosis. At the present time that stage has not been reached, and the possibility that agents other than adenovirus type 8 may be responsible cannot be discarded.

This problem is particularly acute in areas where high endemic levels of epidemic keratoconjunctivitis persist. In Japan, Mitsui has described the "infantile" form of epidemic keratoconjunctivitis as a systemic illness with pseudomembranous conjunctivitis *without* keratitis.[1] This syndrome in individual children may be difficult to distinguish from adenovirus type 3 infection appearing as "pharyngoconjunctival fever." Yet Mitsui has been able to predict from the clinical picture with considerable accuracy whether the infection was caused by adenovirus type 3 or type 8. Serologic study of paired sera from such cases in our laboratory has sup-

ported his diagnosis in most instances.

That epidemic keratoconjunctivitis is a distinct clinical entity is supported by epidemiologic and clinical data. Certainly, wide differences between the epidemiology of pharyngoconjunctival fever and epidemic keratoconjunctivitis have been observed in this country. Pharyngoconjunctival fever, for example, is known to have occurred in the United States, sporadically and epidemically, for many years, while the evidence is strong that epidemic keratoconjunctivitis did not appear here until World War II, when in 1941 it was brought from Hawaii to the Pacific Coast. The propensity of pharyngoconjunctival fever to infect children, to spread in swimming pools, and to appear in late summer and fall, is not shared by epidemic keratoconjunctivitis, while the propensity of epidemic keratoconjunctivitis to spread in clinics and doctors' offices, and to infect doctors and nurses, is not shared by pharyngoconjunctival fever or other types of viral keratoconjunctivitis, such as herpetic keratoconjunctivitis or trachoma.

It seems inevitable that borderline cases will exist, but careful clinical analysis may sometimes resolve the diagnostic problem. One distinctive feature of epidemic keratoconjunctivitis to which insufficient diagnostic importance has been attached in the past is the seven- to 10-day lag between the onset of the conjunctivitis and the development of the subepithelial corneal infiltrates. A comparable lag between conjunctival signs and corneal signs, if any, has not been observed in pharyngoconjunctival fever or other types of viral keratoconjunctivitis. Another feature of epidemic keratoconjunctivitis not shared by pharyngoconjunctival fever is the high incidence of conjunctival pseudomembranes; they have been described in at least one third of reported epidemic keratoconjunctivitis cases, but never in reported pharyngoconjunctival fever cases. Epidemic keratoconjunctivitis is definitely a more prolonged disease than pharyngoconjunctival fever, and, except in infants, as reported by Mitsui,[1] is unaccompanied by pharyngitis or fever. The incidence of keratitis, moreover, is very high in epidemic keratoconjunctivitis and very low in pharyngoconjunctival fever. These differences have for the most part obviated clinical confusion between the two entities, but whether or not they are etiologically distinct has yet to be determined.

2. In a number of documented cases herpes-simplex virus has been isolated from patients diagnosed epidemic keratoconjunctivitis.[23, 24] At times this might have been due to clinical confusion between primary herpetic keratoconjunctivitis and epidemic keratoconjunctivitis. Other instances have been recorded, however, in which an antibody titer rise to adenovirus type 8 was associated with isolation of herpes-simplex virus.[4] Apart from clinically typical epithelial herpetic lesions, herpes-simplex virus is not often isolated from inflammatory lesions of the eye.[25] Might there be some connection between the presence of herpes-simplex virus, adenovirus type 8 infection, and the occurrence of full-blown epidemic pharyngoconjunctivitis with opacities? Up to the present time we have not found a patient with typical epidemic keratoconjunctivitis whose serum did not possess antibodies to herpes-simplex. Thus we are unable to exclude the possibility suggested above.

3. The occurrence of epidemic keratoconjunctivitis in large epidemics in shipyards and other industries has suggested that irritants, chemical and physical, might play a role in the genesis of the disease. It must be remembered, however, that outbreaks also have occurred in eye hospitals and ophthalmologists' offices,[26–28] and that the very frequency of eye irritation in industry leads to frequent visits to the ophthalmologist. Trauma associated with ophthalmologic examination (for example, tonometry) and infection arising from ophthalmologic solutions, materials, or the physician's hands must be considered strongly.

The volunteer studies, up to the present time, have not unequivocally answered the

question of the need for trauma in addition to the presence of adenovirus type 8 to produce clinical epidemic keratoconjunctivitis. While preliminary scarification of the conjunctiva induced "takes" of adenovirus type 8 infection more regularly in volunteers, simple dropping of infected fluid into the eyes also resulted in some infections. Much further study will be required to elucidate this aspect of the problem.

Summary

Since 1951 epidemic keratoconjunctivitis occurring in various parts of the world has been constantly associated with infection by adenovirus type 8. Evidence has been presented that this association involves an etiologic relationship and that adenovirus type 8 is capable of producing a disease compatible with typical epidemic keratoconjunctivitis. It is not implied that it is the sole etiologic agent of this infection. However, current evidence warrants the suggestion that the clinical diagnosis of epidemic keratoconjunctivitis be confirmed by laboratory tests for infection with adenovirus type 8. Additional problems are discussed which will have to be solved before adenovirus type 8 may be accepted as the sole etiologic agent of epidemic keratoconjunctivitis.

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Discussion

Dr. Alson E. Braley (Iowa City, Iowa): It is a pleasure to have the opportunity to discuss such a complete study of the isolation of a virus by modern methods. This virus has been isolated in several locations in California, in Japan, and in Scotland; and all three of these isolations, as I gather it from the paper, are identical.

The neutralizing antibodies have shown a distinct rise in titer, which would mean that this undoubtedly is the cause of epidemic keratoconjunctivitis. The classification of the virus as a type 8 adenovirus, of course, might be questioned, since it does not seem to have any association with any of the other adenoviruses, although there may be some slight cross-agglutination.

I would like to know when the highest titer of the sera develops. Is it at 60 days, 90 days, or when? Most of the paired sera were taken either in a short period during and after the infection, or they might have been taken a year or more later. I wonder if the authors had any evidence that any immunity developed which was of lasting value.

During the 1941-1943 and later in the 1947 epidemics, a number of patients who had had epidemic keratoconjunctivitis in 1941 had recovered from the disease and then, in 1947, a few of these same patients developed epidemic keratoconjunctivitis with corneal opacities. I had felt that as long as the corneal opacities were present, antibodies could be demonstrated.

The study of human volunteers is extremely interesting. I should have liked to have had an opportunity to examine some of them because they present findings similar to those of the human volunteer who was inoculated in 1942, at which time a much milder disease developed than developed with accidental inoculation of the patient.

I wonder if Dr. Mitsui has any further information on these human volunteers, and also whether it was possible to re-isolate the virus from these human volunteers after they had been inoculated.

This is another advance in the study of virus diseases. The paper is well conceived, well presented, and there is very little in it to find fault with.

Dr. David G. Cogan (Boston): I should like to ask Miss Hanna if all these known cases of adenovirus type 8 were bilateral involvements and, if not, whether she has any evidence as to the causes of the unilateral counterpart, wherein it appears that the same clinical entity involves only one eye.

Miss Lavelle Hanna (closing): I am afraid I can't answer your question, Dr. Cogan. I do not know whether there were any unilateral cases in this series or not. In many instances in these isolations from Japan we have not seen the patients. The cases I know were bilateral and, in most instances, the volunteers Dr. Mitsui inoculated had a bilateral infection. I can't say whether there was any difference as far as unilateral and bilateral cases are concerned.

To answer some of Dr. Braley's questions, an adenovirus by definition is one which presents a typical kind of cytopathogenic effect in epithelial cells in tissue culture, and which has a complement-fixing antigen, that is an antigen in common with all adenoviruses. By now there are 16 or so, I don't know the latest count. They all fix complement equally, or nearly so, and are identified as being capable of showing antibody increases in complement fixation between paired sera.

The typing was done at the National Institutes of Health by Dr. Huebner's group, and as far as we are concerned we must accept this virus as being an adenovirus. Since it was the next one in line, it became type 8.

As far as our immunologic studies are concerned, we do not know whether neutralizing antibodies have any clear-cut effect in terms of immunity and protection. However, we do have two instances of volunteers in whom no infection could be produced, and who had neutralizing antibodies at the time of inoculation.

Viruses were re-isolated from volunteers. It showed on the chart but the chart was not very readable.

I would like to ask Dr. Braley if a keratitis was produced in the volunteer he inoculated. I believe there was keratitis in three of the five volunteers of Dr. Mitsui.

PRECIPITATING ANTIBODY TO TOXOPLASMA*

A FOLLOW-UP STUDY ON FINDINGS IN THE BLOOD AND AQUEOUS HUMOR

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In a previous report,[1] a method for the detection of anti-Toxoplasma precipitins in the blood and aqueous humor of patients suspected of ocular toxoplasmosis was described. These antibodies appeared to be heat-stable gamma globulins which gave rise to a single homogeneous band of precipitation when reacted with their homologous antigen in a semi-solid agar gel.

The method of Ouchterlony[2] as modified by Halbert[3] seemed to be most useful in the detection of small quantities of antibody, such as are present in the aqueous humor. By means of the so-called "reaction of identity" (fig. 1) in which bands of precipitation from adjacent pairs of antigen and antibody wells fuse to form an obtuse angle, instead of crossing at their point of natural intersection, it has been shown that there is at least one type of antibody molecule which is common to both aqueous humor from patients with presumptive toxoplasmosis and the sera of infected rabbits.

In each of the four previously reported positive cases, where precipitins were demonstrated in the aqueous humor, the Toxoplasma dye test was positive on the patient's serum at a titer of at least 1:1,024. Similarly, in every case where precipitating antibodies were identified in a given specimen of either aqueous or serum, the Toxoplasma dye test, performed simultaneously on the same specimen, was positive to at least some degree.

In the interval between the first report[1] and the present article, major efforts have been directed toward: (a) the refinement of antibody-detection techniques with special reference to the purification, concentration, and analysis of the antigen; (b) the accumulation of data from the testing of 63 specimens of human aqueous humor (Part I); and (c) the development of radioactive labelled antibody for studies of localized immune reactions in the eye (Part II).

PART I
MATERIALS AND METHODS

A. ANTIGEN-ANTIBODY STUDIES

Antigens used for precipitin reactions in agar consisted of supernatant fluid from the pooled, centrifuged peritoneal exudates of albino mice which had been infected two

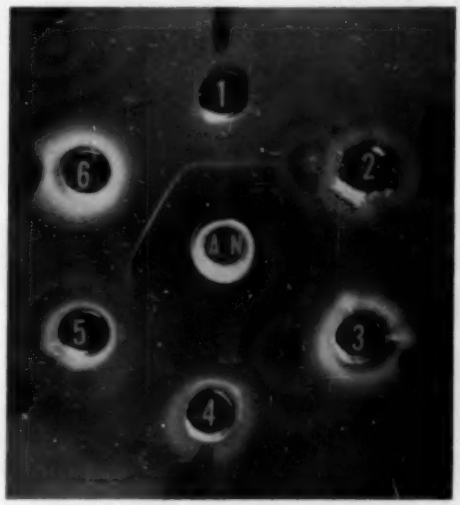


Fig. 1 (O'Connor). Reaction of identity.
AN: Toxoplasma antigen concentrate
1: Rabbit gamma globulin (Dye Test 1:1,024)
2: Human serum: P.M. (dye test 1:256)
3: Human serum: T.B. (dye test 1:256)
4: Human serum: E.E. (dye test neg.)
5: Human serum: N.I. (dye test 1:1,024)
6: Human serum: W.B. (dye test 1:261,504)

* From the Ophthalmology Branch, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

days previously by intraperitoneal inoculation of RH strain Toxoplasma gondii (0.1 ml. of a suspension containing 10^7 to 10^9 organisms [ml.]). The supernatant fluid, originally diluted 1:2 in 0.85 sodium-chloride solution, was then further concentrated by pipetting it into a closed segment of washed dialysis tubing, and allowing it to hang in a stream of cold air at 7°C. for five or six days. In this way, a 10-fold concentration of the original exudate was achieved without appreciable denaturation of the antigen. An alternate method of concentration consisted of dialyzing the supernatant fluid against a five-percent solution of polyvinyl pyrrolidone for 24 hours. This produced satisfactory reduction of the fluid volume in a short time, but apparently denatured the antigen to an extent where it could not be detected.

In another group of four experiments, supernatant fluid from the pooled mouse exudates was diluted 1:1 with cold saturated ammonium sulfate solution. The precipitate obtained from the half-saturated ammonium sulfate solution mixture was centrifuged at 2,800 rpm for 20 minutes. After decanting the supernate, the precipitate was resuspended in an approximately equal volume of distilled water, dialyzed against running tap water overnight, and then against 0.85-percent sodium chloride solution at 7°C. for three days. The soluble and insoluble fractions of the mouse exudate were then tested separately for antigen potency.

Undiluted human and rabbit sera, as well as gamma globulin extracts of the sera, were tested for precipitating antibody, as described previously,[3] using 0.8-percent Difco-Bacto agar buffered with Veronal (pH 7.4) as the diffusion medium. Globulin extracts were prepared both by precipitation in half-saturated ammonium sulfate solution and by means of the electroconvection apparatus of Raymond[4] as described in the previous paper.[1]

B. Aqueous humor studies

Specimens of aqueous humor were tested for precipitating antibody by placing the undiluted aqueous in the central well of an agar plate, and allowing it to diffuse toward six antigen wells, arranged in a hexagon at a radial distance of 1.3 cm. from the aqueous source (fig. 2). Aqueous humor specimens were tested against the antigens of Toxoplasma, Staphylococcus aureus, beta hemolytic Streptococcus (Halbert strain H-414-6),[2] Histoplasma capsulatum, Tubercle bacilli, and a control antigen derived from noninfected mouse tissue, at concentrations of 1.0 mg. antigen protein per ml. When aqueous humor samples containing relatively large amounts of antibody were tested, precipitin lines appeared within 24 hours or less under conditions of refrigeration at 7°C. Where antibody was present in higher dilution, definite precipitation did not become visible before three or four weeks, in some cases.

Results

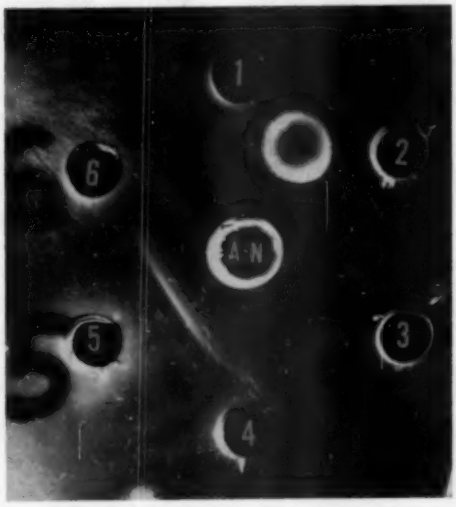
A. Antigen-antibody studies

The antigen solutions, produced by 10-fold concentration of peritoneal exudate, gave rise to much sharper, heavier precipitin lines, when reacted with sera of known high specific activity, than were observed in the original series of experiments.[3] Similarly, more definite lines were observed with aqueous humor from suspect patients when the concentrated antigen was used (fig. 2). As in the case of antigen used for complement fixation tests, the supernatant fluid from the peritoneal exudate of infected mice was a more satisfactory source of soluble antigen than the cellular sediment from this material.

The use of highly concentrated antigens showed, further, that precipitating antibody to Toxoplasma is not a single homogeneous protein, as previously believed, but rather a heterogeneous mixture of at least four precipitating substances, not all of which are demonstrable in every positive serum (fig. 3). Thus when sera from different patients known to be positive for antibodies to Toxoplasma were reacted side-by-side with a single antigen solution of known high po-

Fig. 2 (O'Connor). Precipitins in aqueous humor.
AQ: Aqueous humor sample (F. B.)
1: Toxoplasma antigen
2: Noninfected mouse tissue control (spleen)
3: Staphylococcus antigen (1.0 mg./ml.)
4: Streptococcus antigen
5: Histoplasma antigen
6: Tubercle bacillus antigen (P.P.D.)

tency, a multiplicity of lines was observed, some of which crossed with lines laid down by adjacent sera (fig. 4). It is noteworthy that one single dense band appeared to be



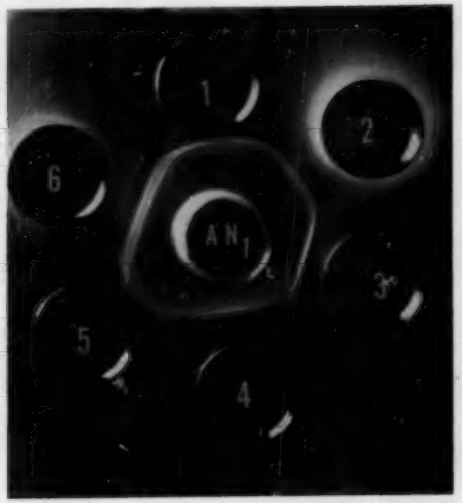


Fig. 4 (O'Connor). Comparison of lines from various sera.
AN_1: Toxoplasma antigen concentrate
1: Human gamma globulin (dye test 1:1,024)
2: Human serum: L.B. (dye test 1:261,504)
3: Human gamma globulin (dye test 1:16,384)
4: Rabbit serum (dye test 1:50,000)
5: Rabbit gamma globulin (dye test 1:1,024)
6: Human serum: J.S. (dye test 1:16,384)

common to all sera, as indicated by the "identity reaction." Whether the additional bands represent immune substances formed in response to particular parts of the organism, as is the case with the somatic and flagellar antigens of the typhoid bacillus, or whether they represent periodic precipitation of various fractions of the same antibody with different avidities for the antigen, remains to be seen.

Fig. 3 (O'Connor). Multiple precipitin lines.
AN: Toxoplasma antigen concentrate
1: Human serum: N.I. (dye test 1:256)
2: Human serum: E.E. (dye test neg.)
3: Ether extract of Serum: E.E.
4: Human serum: R.B. (dye test 1:1,024)
5: Human gamma globulin (dye test 1:16,384)
6: Human serum: E.D. (dye test 1:1,024)

Further protein fractionation studies of the antigen preparations revealed that the antigen was precipitated almost completely in half-saturated ammonium sulfate solution, leaving the albuminous supernate virtually free of activity. The precipitate when redissolved in distilled water and dialyzed against cold normal saline for three days showed undiminished activity, behaving for all intents and purposes like a serum globulin.

B. Aqueous humor studies

Sixty-three specimens of aqueous humor have now been tested for precipitating antibodies to Toxoplasma gondii. The test in its present form is a qualitative one; however, certain semiquantitative observations can be made even under the conditions described. All other factors being equal, the relative density of the precipitin line is proportional to the antibody concentration. As noted above, the time of its first appearance is inversely proportional to its concentration. Its position in the agar with regard to the relative distances between the line and the corresponding antibody and antigen wells is also a measure of concentration; for, if other conditions remain constant, the concentration of antibody at any one point in the agar is inversely proportional to the square of its distance from the antibody source. Since maximal precipitation occurs at a point where the concentrations of antibody and antigen reach a specified optional ratio, it can, in general, be said that a line which appears relatively further away from the antibody well represents a more concentrated source of antibody than one which forms closer to the central well. These observations, combined, permit the grading of the degree of precipitation according to a 1+ to 4+ system.

Of the 63 samples of aqueous humor tested, 32 specimens have come from patients seriously suspected of ocular toxoplasmosis on the basis of high serum Toxoplasma dye tests (over 1:64), positive skin tests, and compatible lesions in the uvea. Three represent samples taken from eyes affected by tumors or other noninflammatory lesions. Eight samples were obtained from patients with negative serum dye tests. The remainder represent specimens procured from patients in whom the evidence for toxoplasmosis, based on standard concepts, was no greater than for other conventional causes of uveitis.

In every case where a sufficient volume of aqueous humor permitted, a Toxoplasma dye test was performed simultaneously on an aliquot of the same sample. The data presented in Table 1 summarize our results to date.

Of the 11 positive specimens, of which one represents a second sample of aqueous, obtained eight months after the first, all came from patients who were strongly suspected of toxoplasmosis on the grounds of positive dye tests, skin tests, and compatible eye lesions. In every case the serum dye test was positive at a titer of at least 1:256. There were no positive reactions among patients with negative serum dye tests, or among patients with dye tests below 1:256, regardless of whether or not their eye lesions seemed compatible with toxoplasmosis.

The aqueous humor from two patients showed precipitating antibodies to beta-hemolytic Streptococcus (Halbert's strain H-414-6). Figure 5 shows a precipitin line between well No. 4 containing streptococcal antigen and the central well containing aqueous humor. One of these patients showed a concomitant antistreptolysin 0 titer of 166 units in her serum.

TABLE 1

STATISTICAL SUMMARY OF PRECIPITIN TEST RESULTS

Total specimens tested	63
Presumptive toxoplasmosis patients	32
Positive toxoplasma precipitin reactions	11
Negative toxoplasma precipitin reactions	52
Uveitis of other possible etiology including nongranulomatous	28
Nonuveitis controls	3
Precipitin reactions to other antigens (streptococcus)	2

TABLE 2

PRECIPITATION REACTIONS IN AQUEOUS HUMOR

Patient	Degree of Precipitation	Serum Dye Test	Aqueous Dye Test	Precipitins in Serum	Flare in Anterior Chamber	Duration of Disease
1. F.P.	++	1:256	—	Neg.	2+	8 yr.
2. W.B.	+++	1:261,504	1:32	4+	3+	7 mo.
3. J.S.	+	1:65,376	1:16	4+	±	3½ yr.
4. N.Z.	+	1:1,024	—	Neg.	±	? 30 yr.
5. R.B.	++	1:1,024	1:64	Neg.	2+	9 mo.
6. T.B.	+++	1:256	1:64	2+	4+	10 yr.
7. E.D.	++	1:1,024	1:64	Neg.	3+	5 mo.
8. F.B.	++++	1:256	1:4	Neg.	3+	6 mo.
9. L.Mu.	+	1:256	1:128	Neg.	2+	4 mo.
10. J.S.	++	1:4,096	1:64	4+	+	4½ yr.
11. L.Mo.	+	1:256	1:256	Neg.	±	1½ yr.

Table 2 characterizes the patients from whom the 11 positive tests for Toxoplasma precipitins were obtained. Serum dye test titers are recorded for each patient, as well as the simultaneous aqueous dye test titer, and the degree of precipitation reaction in both the aqueous humor and the serum. An estimation of the amount of flare in the chamber reaction is also tabulated.

DISCUSSION

On these data several interesting observations can be made. Case 8, for example, from whom the most strikingly positive aqueous precipitin test was obtained (fig. 2), showed an aqueous dye test of only 1:4, and no precipitating antibody in his serum. This same patient had the relatively low serum dye test titer of 1:256. Case 9, on the other hand, who showed a similar pattern of reactions in his serum, had a dye test titer of 1:128 in his aqueous, along with a barely visible aqueous precipitin reaction. From these and other data cited previously,[1] it seems that precipitating antibody and dye test antibody may represent two different molecular entities. That these two substances may appear in different phases of the disease, as is the case with dye test antibodies and complement-fixing antibodies, is likely.

Fig. 5 (O'Connor). Antistreptococcal precipitins in aqueous.
AQ: Aqueous humor sample (L.M.)
1: Toxoplasma antigen
2: Mouse spleen control
3: Staphylococcus antigen
4: Streptococcal antigen (Halbert strain H-414-6)
5: Histoplasma antigen
6: Tubercle bacillus antigen (P.P.D.)

Six cases in the present series showed positive aqueous dye tests at a titer of at least 1:4 without concomitantly positive precipitin tests. The aqueous dye tests, for the performance of which the author is indebted to Dr. Leon Jacobs and Mr. Milford Lundy of the National Institute of Allergy and Infectious Diseases, were positive at a dilution of at least 1:4 in every case of positive precipitin reaction seen thus far. There is, however, little parallelism in the degree of positivity when the two tests are compared side by side.

It would seem that at least a minimally detectable amount of flare must be present in the anterior chamber in order for the precipitin reaction to be positive. This is not to say that granulomatous inflammatory disease of the anterior segment must be present in all such cases. In several instances where only slight flare could be detected, it was felt that this represented a reflection of active inflammatory response in the posterior segment of the eye.

Of particular interest is the fact that seven out of the 11 positive cases have shown precipitating antibody in their aqueous humor, where none could be demonstrated in their serum. Feeling that serum lipids, albumin, or other protein molecules might interfere with the detection of precipitins in the serum, globulin extracts have been made from five of these sera by the ammonium sulfate precipitation method. On testing these globulin extracts, no additional precipitins could be demonstrated. If, as Witmer[5] has intimated in his work on leptospiral uveitis, there is local antibody formation in the uvea, the present studies would tend to substantiate his supposition. The conventional idea that antibodies are found in the eye only by virtue of an increased permeability of the blood-aqueous barrier seems inadequate to explain the findings presented here. Unless, as Verrey[6] suggests, there is stock-piling of antibody in the eye from some previous occasion when precipitins were actually present in the circulation, it must be assumed from these studies that there is local formation of precipitating antibodies in the eye itself. As such, the precipitin test represents a valuable diagnostic aid in the etiologic evaluation of uveitis.

In this regard, the finding of precipitating antibody to beta-hemolytic Streptococcus in two cases of the series presented here supports the previous statement that the technique of testing aqueous by diffusion in agar has general applicability in the sphere of etiologic studies.

Part II

Radioactive labelled antibody

A second category of investigation, involving the use of radioactive labelled antibody, has been undertaken in the attempt to determine the specific nature of a given granulomatous lesion in the eye. On the supposition that a certain amount of Toxoplasma antigen is present in the local tissue site, particularly during the more acute proliferative phase of the disease, lesions should theoretically be identifiable on the basis of the particular antigen detected. If circulating precipitins gain access to the eye at all, it is felt that a relatively larger portion of them should become sessile in the lesion, bound, as it were, to specific antigen.

Materials and Methods

From pooled specimens of rabbit antisera, having Toxoplasma dye test titers of at least 1:1,024, gamma globulin preparations* were made by precipitation in half-saturated ammonium sulfate solution. Ten cc. aliquots of globulin solution, suitably diluted with M/20 Disodium phosphate until they contained approximately 20 mg. of protein per ml., were then labelled with I^{131} in the presence of excess potassium iodide, according to the method of Talmage et al.[7] After the removal of 80 to 90 percent of the non-

* Kindly supplied by Dr. Leon Jacobs, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.

protein bound iodine through the use of an anionic-exchange resin column, the material was sterilized by Seitz filtration. Samples of the preparation were then tested for precipitin activity by the agar-diffusion technique, using a single large well for the antibody solution and another well located eight mm. from it for the Toxoplasma antigen solution (fig. 6).

An attempt was made to demonstrate that the precipitin line so produced was itself radioactive. Accordingly, a glass plate coated with Kodak Photographic Emulsion A (25 μ thickness) was exposed to the surface of the agar for 12 hours under conditions of complete darkness. When the plate, which was separated from the agar only by a single layer of cellophane, was then removed, developed, and fixed, exposure of the photographic emulsion to radioactive particles in the agar could be documented (fig. 7).

Intravenous injections of this radioactive antibody, containing approximately 2.3 μc. per ml. were administered in doses of 2.0 ml. per kg. to a series of eight rabbits. Approximately 0.01 cc. of a suspension of formalin-killed Toxoplasma organisms (containing 0.098 gm. mouse peritoneal exudate sediment per ml.) had been injected, through a No. 27 needle, under the retina of the right eye of each animal 24 hours prior to the administration of the labelled globulin. A similar subretinal injection of a suspension of killed human tubercle bacilli* (2.5 mg./ml. of saline) was performed with the aid of direct ophthalmoscopy on the left eye of each rabbit.

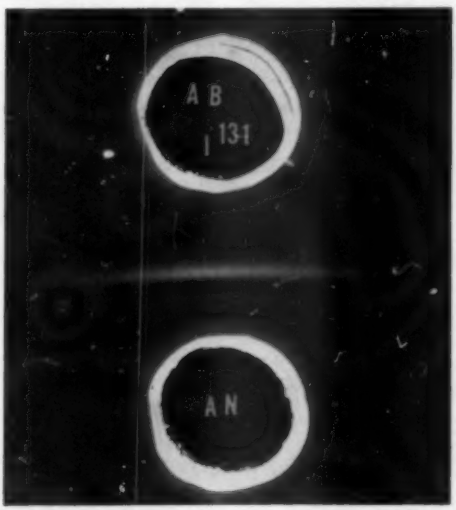


Fig. 6 (O'Connor). Precipitin lines from labelled antibody.
AB I^{131}: Rabbit gamma globulin labelled with I^{131}
AN: Toxoplasma antigen concentrate

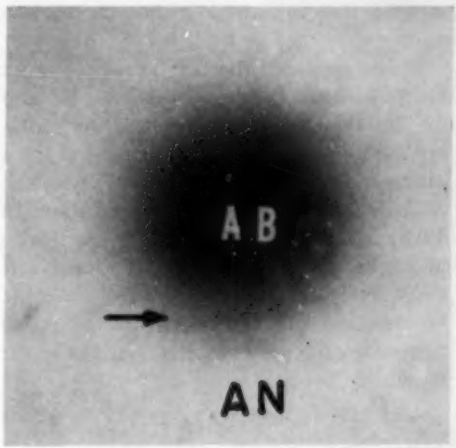


Fig. 7 (O'Connor). Autoradiogram of precipitin line.
AB: Radioactive rabbit globulin
AN: Toxoplasma antigen concentrate
Arrow marks location of precipitin line image

The rabbits were killed approximately 24 hours after the administration of the labelled globulin, at which time five percent of the initially injected radioactive material could still be detected in the blood stream. The eyes were removed immediately and dissected free of the majority of their muscle and adherent tissues. They were rendered soft by paracentesis of the anterior chamber in order that they might fit more easily into the narrow confines of a well-counter, in which as-

*Kindly supplied by Dr. Jules Freund, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.

says of their individual radioactivity were made.

Following this procedure, the eyes were fixed in formalin and then sectioned by both the frozen-section technique and the standard paraffin method. After sections of 25 μ average thickness were cut and mounted, radioautographs were made using standard 1.0 by 3.0-inch microscope slides coated with photographic emulsion A. Additional sections were stained with hematoxylin-eosin, Giemsa, and Kinyoun's carbolfuchsin stains to confirm the presence of organisms in the tissue.

Results

Figures 6 and 7 confirm the fact that precipitating antibody to Toxoplasma can be labelled with radioactive iodine without loss of immunologic activity. The radiographic image of the precipitin line seen in Figure 7 corresponds exactly to the position of the line seen on the ordinary-light photograph (fig. 6), showing that the precipitin line itself is radioactive. It has in it a relatively higher concentration of radioactive particles than the agar immediately surrounding it.

The results of the radioactivity assays on the eight pairs of eyes described above are summarized in Table 3. The values showed no statistically significant difference between the radioactive uptake of the two eyes of a given animal in this series.

Radioautographs of sections of the eyes, developed three weeks after exposure, failed to show selective ocular uptake of radioactive material, although ordinary histologic staining of these sections confirmed the presence of organisms in the retina and choroid, with small numbers of inflammatory cells about them. This preliminary experiment, performed in conjunction with Dr. James O'Rourke and Mrs. Eleanor Collins, of the Ophthalmology Branch of the National Institute of Neurological Diseases and Blindness, would indicate that either circulating radioactive antibody does not gain access to the eye in sufficient quantity to be detected by the methods employed here, or that it cannot displace nonlabelled sessile antibody which may already be present in the tissue.

TABLE 3

COMPARATIVE GROSS RADIOACTIVITY (MC.) OF RABBITS' EYES 24 HOURS AFTER INTRAVENOUS LABELLED GLOBULIN INJECTION

Rabbit No.	Right Eye (Toxoplasma lesion) μc.	Left Eye (Tubercle bacillus lesion) μc.
I	0.00127	0.00127
II	0.00128	0.00177
III	0.00158	0.00126
V	0.00178	0.00198
VIII	0.00164	0.00119
X	0.00180	0.00186
XI	0.00137	0.00167
XII	0.00139	0.00125

Summary

1. The presence of anti-Toxoplasma precipitins in both the blood and aqueous humor of patients suspected of ocular toxoplasmosis has been established.

2. Precipitating antibody has been detected in the aqueous humor of 11 patients out of a total of 32 seriously suspected of ocular toxoplasmosis. The occurrence of precipitating antibody in the aqueous humor of seven patients who had no demonstrable precipitins in their blood suggests that the antibody is formed in the eye itself. Under the conditions outlined, therefore, the precipitin test is of value as an adjunct to diagnosis.

3. Precipitating antibody to Toxoplasma has been labelled with radioactive iodine without loss of immunologic activity.

4. The specific uptake of labelled antibody in eyes with experimental toxoplasmic lesions could not be demonstrated in preliminary experiments.

Ophthalmology Branch (14).

I wish to thank Dr. Leon Jacobs, Dr. James O'Rourke, Mrs. Eleanor Collins, and Mr. Lee Caldwell for their advice and assistance in Part II of this work.

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Discussion

Dr. Alan C. Woods (Baltimore): A study of the precipitin content of the aqueous presents certain technical difficulties which heretofore have been virtually insoluble. The standard microchemical technique of quantitating precipitins is inapplicable on account of the minute quantity of aqueous available. The end-point technique, wherein standard amounts of the fluid to be examined are exposed to diminishing amounts of the antigen, cannot be used to determine aqueous precipitins for the same reason. The standard tube-dilution technique is unsuited for precipitin studies for the reason that when diminishing amounts of the fluid to be tested are exposed to standard amounts of antigen, the inhibitory effect of excess amounts of antigen becomes evident when serum is diluted over 1:20. In the case of the aqueous the initial protein content is so low that precipitins cannot be demonstrated by this technique even in the low dilutions. Dr. O'Connor has now very happily solved this question for us by applying the agar-diffusion technique, and has really opened up a new avenue for investigation in ocular immunology.

In his first paper he reported that the sera of immunized rabbits with a positive Toxoplasma dye test in a titer of 1:50,000 or more, showed specific Toxoplasma precipitins in serum dilutions as high as 1:16. He then outlined the manner in which the agar-diffusion technique could be applied to a study of the specific Toxoplasma precipitins in the aqueous and blood serum both of patients and experimentally immunized animals. In his present paper, he has outlined his further experiences. In summary, he has (1) given us a new method for refining and concentrating the antigen, (2) has reported that in 11 of 32 suspected cases of ocular toxoplasmosis he has been able to demonstrate specific Toxoplasma precipitins in the aqueous, (3) that there is some evidence these precipitins differ and may arise from different fractions of the antigen, (4) that in the over-all picture these precipitins appear to be more abundant in the aqueous than in the blood serum, (5) that there is a marked difference in the relative intensity of the precipitin content and the dye test titer, (6) that these differences suggested to him that the precipitating antibody and the dye test antibody are different immunologic entities, and (7) that the total accumulated evidence supports the tentative proposition of the local formation of antibody in the tissues of the eye.

I believe his findings fully support the hypothesis that the precipitating antibody and the dye test antibody are different immunologic entities. If the precipitating antibody is in truth the sensitizing antibody, its occasional relatively high concentration in the aqueous and ocular tissues may explain some of the peculiarities of ocular toxoplasmosis, notably the acute anterior inflammatory reaction and the intense generalized retinal and subretinal edema occasionally seen superimposed on an old focal glial scar. Both are almost certainly allergic reactions, and are probably dependent on sensitizing antibodies in the ocular tissues.

The question of local antibody formation in the eye is one of intense interest. Certainly such a hypothesis is the logical explanation for the focal reaction to tuberculin which can be produced in the tuberculous eye of both man and experimental animals. In such individuals or animals the systemic administration of an excessive amount of tuberculin will evoke a transient, but violent focal inflammatory reaction in the eye, without any concomitant evidence of a systemic allergic reaction. If one presupposes that the prior ocular disease has left behind highly sensitized ocular tissues as compared to the other body tissues, then this reaction can readily be understood. But the idea of local antibody formation has been an anathema to pathologists. Rich and Follis, in their study of focal reactions in animals with an old tuberculous keratitis, came to the conclusion that the enhanced corneal reactivity to tuberculin was due to the increased vascularity which resulted from the prior tuberculous disease. To my mind, the local production of sensitizing antibodies is a much more logi-

cal and satisfying explanation. I am delighted to see Dr. O'Connor produce experimental evidence supporting this hypothesis.

I must confess I am not unduly surprised at Dr. O'Connor's failure to demonstrate a mobilization of radioactive antibodies around a formalin-killed Toxoplasma antigen injected under the retina of normal rabbits. There are too many unknown potentials which may enter into the question of antibody-antigen union—opsonization, phagocytosis, and so forth. Could the Toxoplasma antigen in this case have been denaturized by the formalin treatment? It was an interesting experiment, and a point I feel confident Dr. O'Connor will ultimately demonstrate in some manner. However, I cannot help but wonder if such antibody accumulation could not be better demonstrated by the injection of tagged antigen in an experimental animal with the specific ocular disease. Thus if ocular toxoplasmosis were produced in a series of hamsters after the method reported by Frankel, and these animals were later injected with a tagged antigen (fluorescein? radioactive iodine?), the actual antibody-antigen union and any excess antibody accumulation in the eye might thus be demonstrated. There might be serious technical difficulties in tagging the antigen, but such an experiment would be most interesting. Were I younger and more energetic I would be tempted to tackle it myself!

One other point, suggested but not emphasized by Dr. O'Conner, has a most tantalizing attractiveness. This point is that this same diffusion agar technique might well be used to demonstrate aqueous precipitins specific for other pathogenic agents known to cause uveitis. Thus one might visualize the etiologic diagnosis of granulomatous uveitis to be reduced to the inoculation of one agar plate from the central well of which aqueous precipitins could diffuse out to meet specific antigens emanating from a surrounding circle of accessory wells containing various antigens. Thus it would only be necessary to obtain a sample of the aqueous, inoculate the center well with it, and the surrounding wells with the specific antigens, to then incubate the plate in a refrigerator for a few hours or a couple of days, then read the lines of specific precipitation and obtain the answer! The same technique could conceivably be extended to determine a specific bacterial sensitivity in nongranulomatous uveitis. Thus an endless amount of labor, much of which is futile, might be spared to the ophthalmologist. However, much as I might wish such a happy development, I doubt that such a "Utopia of diagnosis" will crystallize into reality, but such a test may well develop into a valuable adjunct or confirmatory procedure in difficult and obscure cases.

Dr. O'Connor is to be congratulated on his investigations, his conclusions, and his presentation. He has given us a practical method for the determination of precipitins in the aqueous, has made a valuable contribution to our knowledge of ocular toxoplasmosis, offered evidence to support a radical, but plausible, immunologic concept, and has suggested a probable application of this agar-diffusion technique to the etiologic diagnosis of uveitis. We are in his debt.

DR. MICHAEL HOGAN (San Francisco): I have had some personal communications with Dr. O'Connor, and several months ago had the opportunity to see his work at the National Institutes of Health. Last summer we had a summer student fellow working on this technique, and after personal correspondence with Dr. O'Connor we got a fair amount done.

Our difficulties seemed to lie in inability to get clear-cut lines on performing the test. Also, the lines which did form took a considerable period to develop, sometimes as long as four to six weeks. However, I think our difficulties were probably the same as those Dr. O'Connor had when he started doing the test, and I believe his ability to develop a concentrated antigen along with refinements in the type of agar used have made this an extremely valuable tool for the investigation of certain ocular immune mechanisms.

The one aspect of this problem that bothers me is that, ordinarily, in a patient with ocular toxoplasmosis, the disease in most instances affects the posterior choroid and retina. I wonder how it is possible for such a disease to cause a high rise in antibody titer in the aqueous humor.

Normally the flow of fluid, I believe, from the posterior eye is outward through the adjacent retina or through the optic disc. One would expect, if an antibody diffused from the acute lesion, that it would diffuse toward the posterior eye rather than the anterior eye.

I suppose one explanation would be that, somehow, the antibodies got into the aqueous humor via the circulation of the anterior eye. One explanation, as emphasized by Dr. O'Connor, is that actual local antibody formation occurs in the anterior eye in response to the disease.

Those of you who saw Mrs. Wilder's preparations will remember that there was quite a reactive inflammation in the ciliary body and iris in some of the cases; however, in others it was insignificant. This might explain the variation in precipitin titer in some of Dr. O'Connor's cases.

One of his patients had a low serum dye test titer, 1:64, I believe, and a four-plus precipitin titer in the aqueous humor. I think his explanation of a possible local rise as a result of previous infection is likely, because we (and Dr. Woods also) have found cases in which a relapse of an ocular disease resulted in a very high serum dye test titer, out of all proportion to the titer one might expect with such a small degree of ocular inflammation. In two of our cases in which such a relapse occurred, the dye test titer was 1:16,000. If this can occur with serum antibody it should occur locally.

So, I believe the eye and the serum probably are capable of reacting much more strongly in patients who have had previous infection, and this would account for such a result.

DR. G. RICHARD O'CONNOR (closing): I will make my closing remarks very brief. I think Dr. Hogan's cogent comment about the absence of anterior segment response is the most important thing that has been mentioned. Unfortunately, if we don't find some degree of flare in the anterior segment, we get no precipitin line. We are dependent on that for positivity of our results.

Just how the antibody reaches the anterior segment remains somewhat of a mystery, but I think its presence is common, as shown by Mrs. Wilder's findings of inflammatory cells, particularly plasma cells, in the ciliary body and iris root of eyes in which toxoplasmosis was identified, which suggests that most likely there is antibody formation in the anterior segment of the eye.

Dr. Woods' suggestion that antigen be labeled and injected into the eye is a very good one. However, that presents almost insurmountable technical difficulties because the antigen is difficult to obtain in a truly pure form. There always is some protein from the mouse host from which the organisms are originally obtained; at the moment that is the principal difficulty with his suggestion.

I might comment that the eyes can be shown to have some radioactive material in them by exposing sections to photographic emulsion on glass plates. This happened in eyes which were injected with tubercle bacilli and Toxoplasma. However, we could show no differential uptake between the Toxoplasma eye and the tubercle bacillus-injected eye.

THE ROLE OF INTRAOCULAR PRESSURE IN THE DEVELOPMENT OF THE CHICK EYE: III. CILIARY BODY*

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INTRODUCTION

There is a wealth of evidence that during development the expanding vertebrate eye exerts a mechanical influence on the morphogenesis of surrounding structures. The role of the eye in orbital development has been studied by Wessely (1920), Coulombre and Crelin (1957), Washburn and Detwiler (1943), and others. Amprino (1949, 1950, 1951) has extensively evaluated the role of the growing eye in the development of the adnexa.

Less experimental work has been done on the role of intrinsic mechanical factors in the morphogenesis of the eye itself. Weiss and Amprino (1940) and Amprino and Pansa (1955) have evaluated the role of intraocular pressure in the shaping of the scleral cartilages in the developing eye of the chick. Coulombre (1956, 1957) has reported on the role of intraocular pressure in the growth of the eye as a whole and of the cornea in particular. The regular geometry of the eye and its parts makes it most suitable for a detailed quantitative analysis of the role of mechanical factors in morphogenesis.

In the present report a descriptive study has been coupled with the experimental approach in an attempt to discover some of the factors which mold the developing ciliary body.

MATERIALS AND METHODS

The chick embryos used were from eggs of a Cornish-Rock cross, which were incubated at 37.5°C. in a forced draft incubator. All ages are given as days following the onset of incubation.

A quantitative description of the development of the ciliary body and its surrounding tissues was made with normal specimens. All of the observations and measurements mentioned below were made on a minimum of 10 eyes at each day between the sixth and the 25th. Linear measurements were done on dissections of freshly enucleated eyes, with a calibrated filar ocular micrometer fitted on a stereomicroscope. In this manner the diameters or widths of the lens, ciliary body,

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and iris were obtained. Counts of the ciliary processes included secondary as well as primary processes. To measure the area of the ciliary body photographs of the internal aspect of the anterior segment of freshly dissected eyes were taken at known magnification. The negatives so obtained were enlarged 10 diameters with a photographic enlarger and traced on paper. On such tracings planimetric measurements were made of the area occupied by the image of the ciliary zone. The results were corrected for total magnification to yield the true area of the ciliary zone. In both the micrometer measurements and the photographic procedure care was exercised to minimize parallax.

The role of intraocular pressure in the morphogenesis of the ciliary zone was demonstrated experimentally. A previously described microsurgical procedure (Coulombre, 1956) was used to reduce intraocular pressure at four days, the time at which the ciliary zone first appears. Briefly, one end of a short length of microcapillary glass tubing was introduced through punctures made in the eye walls of the right eyes of four-day embryos. These tubes remained in place for the duration of the experiment to allow continuous egress for vitreous humor. The experimental animals were killed for study at either seven, 10 or 17 days. On each of these days the animals were divided into two groups. The eyes of the first group were measured in the manner described above for normal eyes. From the second group both the experimental right eyes and control left eyes were fixed in Zenker's-acetic solution, embedded in paraffin, serially sectioned at 5.0 μ in either the axial or equatorial plane, and stained with hematoxylin and eosin.

In a separate group of five embryos the right eyes were intubated on the fourth day, but the tubes were removed 24 hours later. At nine days the eyes were dissected in the fresh condition and measured as above. No specimens of this group were sectioned.

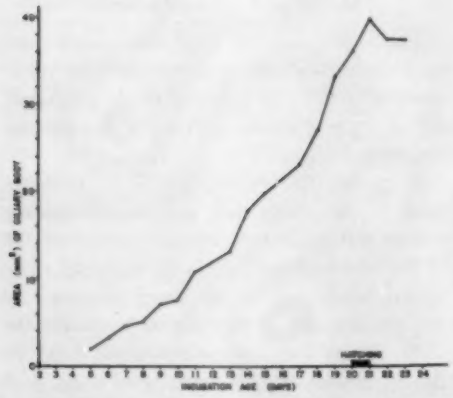


Fig. 1 (Coulombre and Coulombre). The area of the ciliary body as a function of age. Each point represents the mean of measurements made on 10 specimens.

Results

Descriptive study

On the fourth day the edge of the undifferentiated optic cup is in intimate contact with the lens equator. Grossly there is no hint of a ciliary zone, and the iris has not yet appeared. On the fifth day the inward migration of melanin granules in the outer layer of cells of the future ciliary zone make this region visible grossly as a darker ring between the retinal region of the optic cup and the lens equator (Coulombre, 1955). The prospective ciliary zone is firmly attached to the lens equator at all points, and the foldlike ciliary processes are not yet in evidence. From the time of its appearance until the eighth day this zone undergoes a steady increase in diameter and area (fig. 1) but remains symmetric about the lens axis (fig. 2).

On the eighth day marked changes are initiated in the anterior segment of the eye. The first of these is the differentiation of scleral cartilage just outside the corneal limbus in the region overlying the now closed choroid fissure (fig. 3). From this point the cartilagenous differentiation spreads around the corneal margin in both directions until, by the ninth day, a ring of scleral cartilages has been formed. Simultaneously with the

appearance of the scleral cartilages the eye, hitherto spherical, bulges with greater curvature anteriorly to the differentiating cartilage and, on the eighth day, the corneal bulge becomes distinct for the first time (fig. 3).

As these events occur, the first of the radially arranged ciliary folds appear. These folds develop first in the anterior-inferior rim of the optic cup just below the closed choroid fissure, in the same area in which the sclera is forming cartilage (fig. 3). As the eighth day progresses more folds appear one at a time in an orderly progression around the circumference of the cup. By the time that the folds inferior to the choroid fissure have progressed one quarter of the way around the cup margin, additional folds appear superiorly to the choroid fissure and progress in the opposite direction.

The processes continue to increase in number and in length until, by the end of the eighth day, the entire ciliary body becomes plicated with about 70 radially oriented folds. On the ninth and 10th days about 20 additional secondary folds appear between some of the primary folds, bringing the total number to an average of 90 (fig. 5). The number of folds does not increase thereafter, but their depth continues to increase as the eye grows. The tips of the processes thus formed adhere to the lens capsule. Interstices appear between the tips and put the vitreous chamber into communication with the future aqueous compartments in front of the lens (fig. 4a).

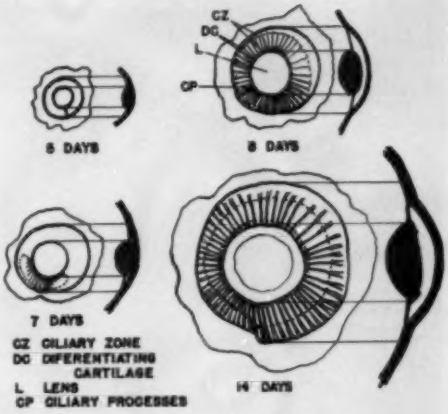


Fig. 3 (Coulombre and Coulombre). Diagrammatic posterior and axial section views of the anterior segment of the developing chick eye at different ages.

Concomitantly with the first appearance of the corneal curvature and the formation of the first ciliary processes, the expansion of the ciliary zone becomes asymmetric. Hitherto of equal width at all points, it now expands relatively more slowly in the anterior ventral quadrant where the scleral cartilages have begun to differentiate. As a consequence the geometric center of the ciliary zone no longer coincides with the corneal-lens axis, but becomes shifted posterodorsally as time elapses (figs. 3, 6, 7, and 8). In keeping with this development of asymmetry the ciliary processes are narrowest at the slowly expanding anteroventral position of the cup rim, and widest posterodorsally, a condition which persists throughout life. The increase in asymmetry of the ciliary zone is reflected in daily measurement made at the points of its least and greatest width (fig. 2). The ring of scleral cartilages which develops over the ciliary zone is coextensive with it and shares its asymmetric growth.

The iris first appears on the ninth or 10th day as a circular shelf arising from the free

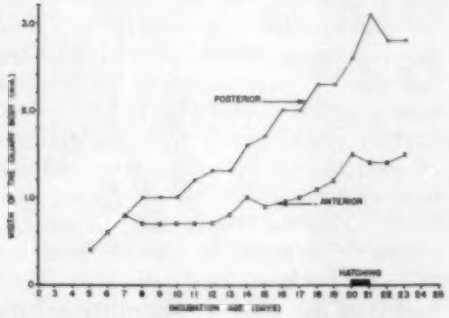


Fig. 2 (Coulombre and Coulombre). The width of the ciliary zone anteriorly and posteriorly as a function of age. Each point represents the mean of measurements made on 10 specimens.

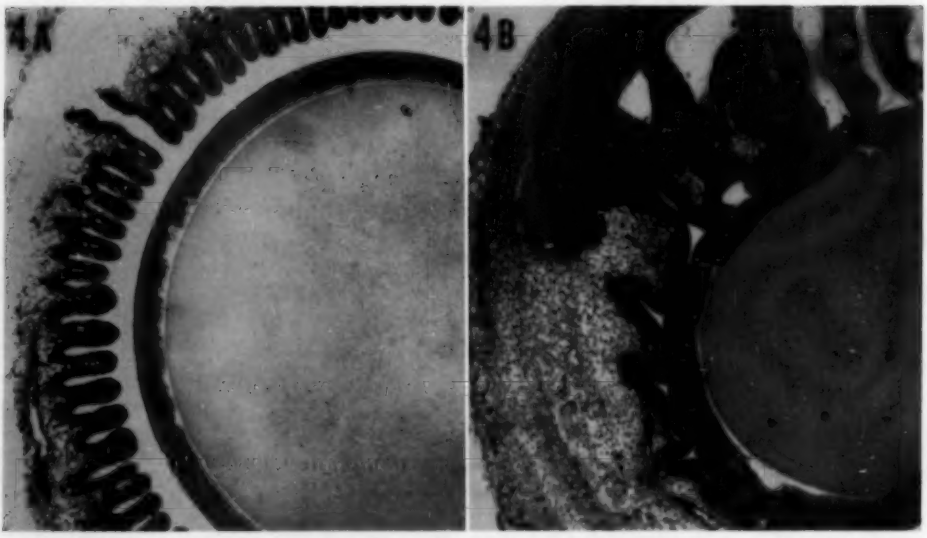


Fig. 4 (Coulombre and Coulombre). (a) Equatorial section through the lens of a normal 10-day-old eye. (b) Equatorial section through the lens of a 10-day-old eye which had been intubated at four days.

edge of the ciliary zone. It increases rapidly in width until the 17th day, after which it is constant in width until at least the 25th day (fig. 5).

Experimental study

Those eyes which were intubated on the fourth day, and in which the tubes remained until termination of the experiment, were examined at different ages either by dissection or in histologic sections. They develop no normal ciliary folds. Instead, the ciliary zone thickens progressively and becomes thrown into relatively large and disoriented folds that are continuous with gross folds in the neural retina (fig. 4). Even in the oldest eyes examined the optic cup remains smoothly adherent to the lens over long distances. In addition, asymmetry develops neither in the ciliary zone nor in the ring of pericorneal scleral cartilages. In the intubated eyes the attachment of the rim of the optic cup to the lens becomes displaced progressively more anteriorly to the lens equator as time elapses (fig. 9).

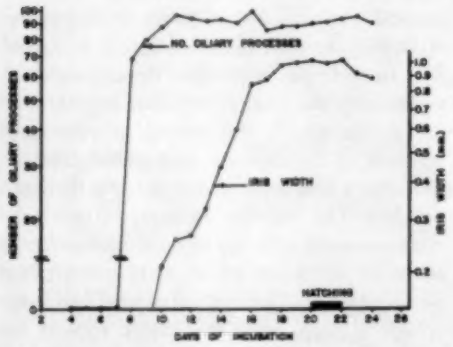
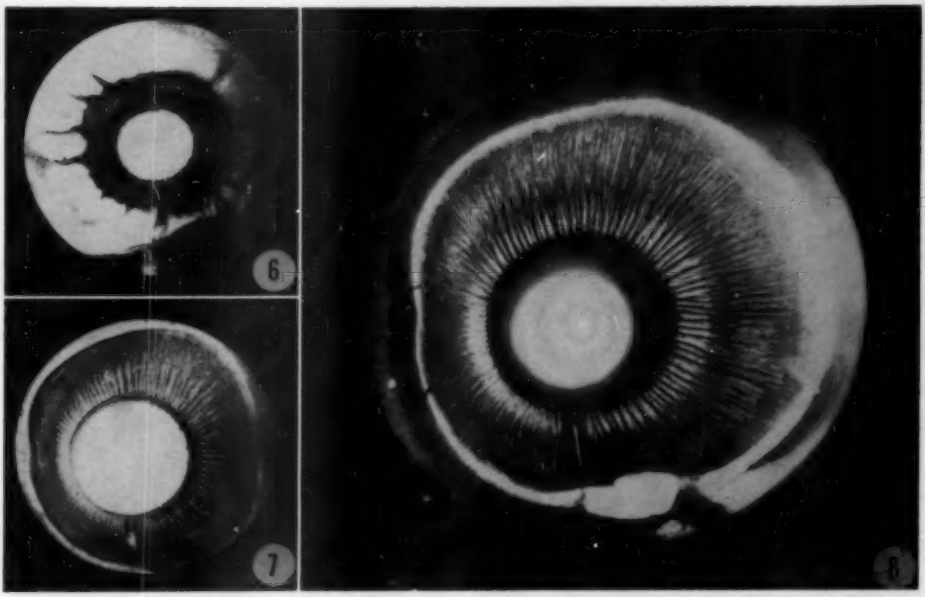


Fig. 5 (Coulombre and Coulombre). The number of ciliary processes and the width of the iris as functions of age. Each point represents the mean of measurements made on 10 specimens.

These effects derive from the drainage afforded by the indwelling tube, and not from operative trauma or other nonspecific factors. This was demonstrated in previous studies in a series of control operations (Coulombre, 1956, 1957), in which small solid glass rods were introduced through the eye wall instead of tubes. These glass rods duplicated all aspects of the intubation operation, with the exception that vitreous humor



Figs. 6, 7 and 8 (Coulombre and Coulombre). (6) Posterior view of a seven-day-old ciliary zone (right eye). Note the incomplete ring of ciliary processes. Photographed by transmitted light. (7) Posterior view of an 11-day-old ciliary zone (right eye). Photographed by transmitted light. (8) Posterior view of an 18-day-old ciliary zone (right eye). Photographed by transmitted light.

could not escape. Such control eyes develop normally in all respects.

In five intubated cases the drainage tube was removed from the eye 24 hours after it had been introduced. In these specimens the eye wall heals, and the eye resumes its increase in size at a rate slower than normal (Coulombre, 1956). These eyes were dissected at nine days of incubation. They were smaller in all dimensions than the contralateral control eyes but appeared to be normally proportioned. They showed a smaller number of ciliary folds and processes than normal.

Discussion

The data reported above suggest that the following sequence of events occurs during the development of the anterior segment of the chick eye. Following the invagination of the optic cup and lens primordium, vitreous humor accumulates between the lens and the presumptive retina. As development proceeds, expansion of the vitreous body places the eye wall under tension and the eye increases in size. The increase in area of the eye wall depends, among other factors, on the imbalance between tangential forces arising from intraocular pressure and the resistance offered to these forces by the eye wall (Coulombre, 1956). Between the fourth and the eighth day the resistance of the eye wall to expansion must change about equally at all points on its surface, since the eye enlarges essentially as a sphere.

On the eighth day, however, changes occur locally in the sclera which render it inhomogeneous and make inevitable a progressive change in the shape of the eye as development proceeds. The most notable change is the appearance for the first time of cartilagenous plates in the sclera (Weiss and Amprino, 1940). Cartilage begins to differentiate just outside the corneal limbus, overlying the closed choroid fissure.

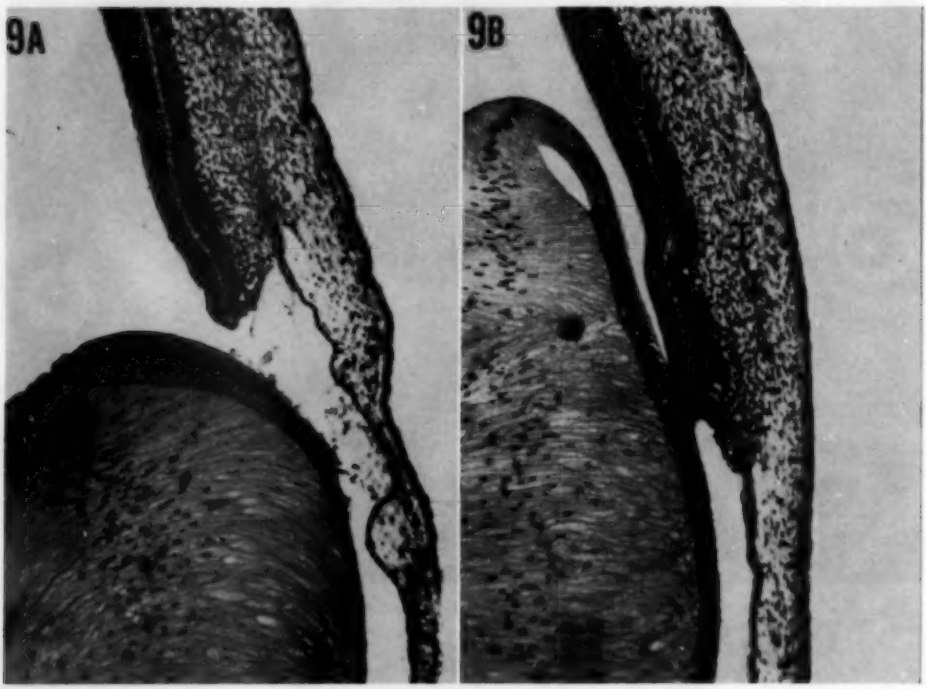


Fig. 9 (Coulombre and Coulombre). (a) From an axial section of a normal seven-day-old eye. (b) From an axial section of a seven-day-old eye which had been intubated since the fourth day. Note the anterior location of the ciliary body-lens junction.

As a consequence this region expands less rapidly than the remainder of the eye surface from the eighth day onward. The cartilagenous tissue spreads around the circumference of the cornea as time goes on, forming a complete ring of pericorneal scleral cartilages by the ninth day.

This ring does not expand as rapidly as the remainder of the eye surface; and from the eighth day the eye develops, under the influence of intraocular pressure, not as a sphere but as two spherical zones (the corneal and the scleral zones) which have a common base bounded by the cartilagenous ring. The radii of curvature of these zones are at first equal but diverge progressively as development proceeds. As it forms, the cartilagenous ring becomes relatively broader in the upper posterior quadrant (where expansion of the eye wall is most rapid), than over the region of the choroid fissure where it first began to form. Thus the ring becomes increasingly asymmetric as time goes on (fig. 3).

The ciliary zone, which underlies the ring of scleral cartilages, reflects the increasing asymmetry of the latter. The ciliary zone first becomes evident grossly on about the fourth or fifth day as a darker ring at the edge of the optic cup, attached anteriorly by its smooth free edge to the lens equator, and continuous posteriorly with the retinal portion of the cup. From this time until the eighth day it is radially symmetrical around the lens axis. Beginning on the eighth day, however, it expands much more rapidly toward the upper posterior quadrant than it does toward the lower anterior quadrant where cartilage is differentiating in the sclera.

When intraocular pressure is reduced or eliminated by intubation, both the ring of scleral cartilages and the ciliary zone remain symmetric about the lens axis. This suggests that the asymmetric expansion results from tangential forces generated equally at all points on the eye surface by the expanding vitreous, but counteracted to different degrees at different places by structural elements in the eye wall. Rochon-Duvigneaud (1943) calls attention to similar, but much less marked, asymmetries in the adult human ciliary zone, which may arise in a similar manner.

It has already been noted that the processes of the ciliary body also show a striking correlation in the time and place of their appearance, as well as their pattern of spreading, with the time and place of appearance and the pattern of spreading of the cartilagenous elements in the sclera. It is improbable that these correlations are fortuitous.

It seems probable that mechanical forces, acting in the changing geometric framework of the anterior segment of the eye, are cardinal factors in the folding of the ciliary zone. This view finds some support in the present data.

When the mechanical forces generated by an expanding vitreous are eliminated or reduced by intubation, the ciliary processes fail to develop normally, and are represented by only a few thick and disoriented folds. Thus, while the folds may result when the choroid zone expands in area more rapidly than the overlying sclera, it appears that they owe their regularity and radial orientation to forces generated by intraocular pressure.

In addition to this, it is possible to influence the number of ciliary processes which develop by controlling the geometry of the anterior eye segment at the time they appear. Thus when eyes are intubated at four days and the tubes are removed 24 hours later the eyes expand less rapidly than normal and the ciliary zone, while normal in appearance, develops fewer ciliary folds.

The facts that the ciliary zone is thicker than normal in such eyes and smaller in total area suggest that the number of folds which develop may be a function of the dimensions of the zone and of its elastic properties at the time folding is initiated.

On the basis of these observations it is suggested that the ciliary folds result when and where there is a toughening of the pericorneal sclera which restricts scleral expansion locally. If the subjacent ciliary zone then continues to expand (as it appears to) it would inevitably become folded or thickened, or both, because of the restriction of its freedom to spread. Since the ciliary zone is normally under radial tension as a result of intraocular pressure, and since the resistance developing in the sclera is predominantly circumferential, folds would naturally dispose themselves radially. This hypothesis makes understandable the remarkable temporal and spatial correlations between the spread of cartilagenous differentiation and the progressive addition of ciliary folds.

The first appearance of the iris *after* the development of the ciliary processes and the apparent establishment of channels of communication between the vitreous and aqueous chambers is suggestive. Before the eighth day the edge of the optic cup has been firmly attached to the lens equator at all points and, as a result of intraocular pressure, the ciliary body is under radial tension. It is possible that this condition effectively prevents the outgrowth of the iris shelf anteriorly. On the eighth day the ciliary interstices first appear, mucoid material is first observed in the aqueous chamber, and the ciliary zone becomes more flattened. This suggests that, with the establishment of connections between the two compartments, vitreous humor escapes into the aqueous chambers, thus equalizing pressure on either side of the ciliary membrane and its subtended lens. This might relieve tension on the edge of the optic cup and permit the outgrowth of the iris shelf.

Throughout this study the schedule of

cellular differentiation was neither retarded nor accelerated by the operative procedures used. Thus the mechanical factors with which this study deals can be assumed to influence morphogenesis on the supracellular level without altering patterns of cellular differentiation. The emphasis placed on mechanical factors must not be allowed to obscure other categories of factors (genetic, biochemical, mitotic, and so forth), which, at different levels, contribute to the molding of the eye.

Summary

During early development the ciliary body expands symmetrically around the lens. When cartilage and collagen begin to differentiate in the sclera at the corneal limbus the ciliary body begins to expand asymmetrically, and radially arranged ciliary folds appear rapidly. Observations and experiments suggest that:

a. The ciliary folds appear where and when the ciliary zone expands in area more rapidly than the overlying sclera.

b. These folds owe their radial orientation to forces generated by intraocular pressure.

c. The number of folds developed may be a function, among other factors, of the dimensions of the ciliary body at the time the folds appear.

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Discussion

Dr. George K. Smelser (New York): On my first reading of this paper my impression was of the great number of differences between the development of the chick eye and the mammalian eye.

A second thought (and I think this paper deserves a serious reading) is that these differences which are so obvious are indeed fortunate. I think Dr. Coulombre has been fortunate in selecting the chick for his study. The differences, for example, the heavy cartilage ring in the chick eye and the marked asymmetry of the ciliary body are, I believe, responsible for the success the authors have had in demonstrating the role of intraocular pressure (in this case vitreal pressure, as opposed to the usual concept of intraocular pressure of the adult) in forming the eye.

The mammalian eye has a dense condensation of scleral collagen, instead of the firmer cartilage plaque, which forms first in the limbal area and not so asymmetrically as in the bird. Had an experiment been attempted using the mammalian eye (if such had been possible) I think it is very likely that these striking results of Coulombre's might

not have been so clearly shown. The selection of this particular experimental animal aided in the demonstration of the role of intraocular pressure in the formation of the eye.

Two factors should be pointed out. The first has been emphasized this morning—the mechanical one. The eye is apparently a little balloon which has to be inflated to take its proper form. This would not produce ciliary folds if a second factor, growth, was equal everywhere. In certain areas of the retinal cup there are zones of proliferation as a result of which the tissue (in this case the ciliary epithelial region) becomes redundant. So that in the absence of pressure to inflate the eye properly, it hangs in the irregular folds that you have seen illustrated. We have to consider, then, that the centers of growth plus this inflation of the globe by the intraocular pressure together mold its form.

I would like to mention another paper by the Coulombres that is probably not widely known to this group, since it was published in the *Journal of Experimental Zoology*, **133**:211-225, 1956. The authors did the carefully controlled experiment of intubation as described today. They showed that if the eye is intubated and intraocular pressure not allowed to develop, one does not get a normally formed retina. The retina itself forms in loose folds and in rosettes, thus reminding one of the retinal structure seen by the pathologist in microphthalmus. Retinal rosettes and folds in microphthalmus may be the result of the failure of normal embryonic intraocular pressure to develop.

In the *Archives* (**30**:338-351, 1943) a number of years ago there was an article by Ludwig Browman, who had in his possession a strain of microophthalmic rats in which he studied the development of the abnormal eyes. As nearly as he could determine, the primary failure was in the development of the blood vessels of the vitreous humor. It appears now that what he was really describing was the basis of the shaping and distention of the globe, which has been ably shown today; that is, the growth of the vitreous humor. Browman was mainly impressed by the blood vessels supplying the vitreous humor.

I think this morning we have had an example of a simple, direct type of experiment, which has yielded much information on the "how" of a biologic process. I wish to thank the authors for their very interesting paper, for the opportunity to discuss it, and to express the hope that the two Coulombres will be here again with more of their work.

Dr. Pei-Fei Lee (Boston): I would like to ask what influence high intraocular pressure has on the development of the ciliary process, simply by raising the intraocular pressure to a certain point through the drainage tube. Dolcet (1952) stated: at birth, in human beings the intraocular pressure is very much higher than the adult value, being some 41 to 56 mm. Hg, the pressure falling at a rate of about 1.0 mm. Hg per day to reach what this author considers a normal adult value of 26 to 29 mm. Hg in about four to six weeks. Therefore, if this is true, it would be interesting to know what is the influence of high intraocular pressure on the development of the ciliary process of the chick eye?

Dr. Alfred J. Coulombre (closing): The eyes which we operated on at about four days had a diameter of one mm. Since the eye wall at this time is extremely soft direct measurement of pressure is not feasible and the purported reduction in intraocular pressure is an assumed one. We have demonstrated, however, that fluid, presumably vitreous, does make egress from the eye in the intubation operation.

When we use solid glass rods instead of tubes, thereby duplicating all of the trauma and all of the nonspecific factors, but preventing the escape of vitreous, we get a perfectly normal development of the eye in all respects.

As for direct measurements of the intraocular pressure, it has been impossible to date for us to measure intraocular pressure at very early ages; indeed, we have made no attempt in the later stages of prehatching development, which would be comparable to the postnatal development in many mammals. Until such measurements can be made, we have no answer to the question.

THE EFFECTS OF MATERNAL HYPOXIA ON INHERITANCE OF RECESSIVE BLINDNESS IN MICE*

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The production of congenital abnormalities in the young of many species of animals has been accomplished by treating pregnant females with such agents as irradiations, nutritional deficiencies in pantothenic acid, riboflavin and folic acid, deficiency and excess of vitamin A, cortisone, insulin, nitrogen mustard, reduced atmospheric pressures, and injections of trypan blue dye.[1-8] These investigations have furnished valuable information as to the teratogenic action of the various agents in regard to disturbance of the normal metabolic processes. The mode of action of the teratogenic agent is not the primary interest in this study but rather to test the effects of the agent on the inheritance of a known genetic defect.

MATERIALS AND METHODS

The mice used in these experiments have been maintained as two inbred colonies in this laboratory for the past five years.

1. A colony of congenitally anophthalmic mice having two recessive factors for eyelessness, genotype CCaabbddpp ey-1 ey-1, ey-2 ey-2 (fig. 1). They are bilaterally anophthalmic and in only one instance has a small, nonfunctional eye been observed in the colony.

2. A colony of inbred mice having no known congenital defects (fig. 2). These mice have approximately the same genotype as the blind mice except for the presence of dominant factors for eyes, CCaabbddpp Ey-1 Ey-1, Ey-2 Ey-2. The mice in both colonies are sturdy and prolific, their litters averaging seven or eight young.

Reciprocal matings were made between the two strains of mice in order to establish the fact that the factors for eyes are dominant regardless of whether the recessive trait for anophthalmia is carried by the father or mother. Then 0.25-cc. subcutaneous injections of trypan blue were given to the females of reciprocal crosses once daily on the seventh, eighth, and ninth day of gestation. Inbred and cross-bred females were also given 0.25-cc. injections of sterile distilled water on the same days of gestation; 1.0-percent, 0.5-percent, and 0.3-percent aqueous solutions of trypan blue dye were used. Pregnancy was determined by vaginal smears.

RESULTS

All the females given injections of 1.0-percent trypan blue lost considerable weight within a few days after receiving the third injection and were autopsied. In every case they had either aborted or were in the process of absorbing their embryos. The females that were given injections of sterile water produced young with normal eyes.

The results following injections of 0.5-

Fig. 1 (Barber). Mice whose only known congenital abnormality consists of bilateral anophthalmia.

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Fig. 2 (Barber). Mice having no known congenital anomalies used for cross-breeding.

percent trypan blue are shown in Table 1. The mice in this experiment were killed and autopsied on the expected date of delivery in order to prevent destruction of the young. Five of the 12 females showed evidence of abortion and four showed signs of absorption (fig. 3). Among the 34 feti recovered there were 13 showing craniorachischisis (fig. 4), three had apparently normal heads but were unilaterally anophthalmic, and one normal fetus.

The results obtained from injections of 0.3-percent trypan blue are shown in Table 2. These mice were also autopsied with the exception of two, Z-168 and N-61, that delivered unexpectedly. The fetuses were generally smaller in size and fewer in number than usual and there was a high percentage of absorptions (figs. 3 and 5). Out of 17 pregnancies 43 intact fetuses were recovered. The incidence of craniorachischisis

TABLE 1

0.25-CC. INJECTIONS OF 0.5-PERCENT TRYPAN BLUE GIVEN ON THE 7TH, 8TH AND 9TH DAY OF GESTATION

Mouse	Cross	Litter	Results
S-46	N ♀ × B ♂	3	2 craniorachischisis 1 right eye missing
S-47	N ♀ × B ♂	7	7 craniorachischisis
S-42	N ♀ × B ♂	6*	Partly absorbed
S-45	N ♀ × B ♂	6*	Partly absorbed
S-53	N ♀ × B ♂		Aborted
S-54	N ♀ × B ♂		Aborted
S-51	N ♀ × B ♂		Aborted
S-52	N ♀ × B ♂	3*	Partly absorbed
Z-161	B ♀ × N ♂	3–2*	1 right eye missing 1 left eye missing 1 craniorachischisis 2 partly absorbed
Z-162	B ♀ × N ♂		Aborted
Z-159	B ♀ × N ♂	4	1 normal eyes 3 craniorachischisis
Z-158	B ♀ × N ♂		Aborted
12		17–17*	13 craniorachischisis 3 unilateral anophth. 1 normal eyes

* Not considered because of degeneration.
N—Mouse having normal eyes.
B—Mouse having bilateral anophthalmia.

TABLE 2

0.25-CC. INJECTIONS OF 0.3-PERCENT TRYPAN BLUE GIVEN ON THE 7TH, 8TH AND 9TH DAY OF GESTATION

Mouse	Cross	Litter	Results
Z-168	B♀ × N♂	5 (del)*	Destroyed
Z-169	B♀ × N♂	6	4 normal eyes 1 craniorachischisis 1 right eye missing
Z-170	B♀ × N♂	7*	Partly absorbed
Z-176	B♀ × N♂	6	2 normal eyes 2 right eye missing 2 left eye missing
Z-181	B♀ × N♂		Partly absorbed
N-57	N♀ × B♂	3	1 normal eyes 1 right eye missing 1 left eye missing
N-61	N♀ × B♂	10 (del) (3–7*)	2 normal eyes 1 right eye missing 7 destroyed
P-53	N♀ × B♂	3	2 normal eyes 1 right eye missing
P-52	N♀ × B♂	6*	Partly absorbed
R-18	N♀ × B♂	5*	Partly absorbed
N-45	N♀ × B♂	3	1 eyes (cataracts) 2 craniorachischisis
N-46	N♀ × B♂	6*	Partly absorbed
N-53	N♀ × B♂	7	1 right eye missing 2 left eye missing 4 normal eyes
P-43	N♀ × B♂	5	5 normal eyes
P-46	N♀ × B♂	4	4 normal eyes
P-47	N♀ × B♂	3	1 right eye missing 2 left eye missing
P-48	N♀ × B♂	3*	Partly absorbed
17		43–39*	25 normal eyes 15 unilateral anophth. 3 craniorachischisis

* Not considered because of degeneration.
N—Mouse having normal eyes.
B—Mouse having bilateral anophthalmia.

was reduced to three cases. There were 25 fetuses with normally developed eyes and 15 with unilateral anophthalmia, eight with the right eye and seven with the left eye missing.

The unilaterally anophthalmic female produced by N-61 (table 2, fig. 6) survived and was back-bred twice to the normal line. She produced two litters, a total of 13 young, having normal eyes. She was then back-bred to the anophthalmic strain and produced one litter, nine young, of which two are bilaterally anophthalmic, four are unilaterally an-

TABLE 2-A: CONTROLS

0.25 CC. INJECTIONS OF 0.3-PERCENT TRYPAN BLUE GIVEN ON THE 7TH, 8TH AND 9TH DAY OF GESTATION

Mouse	Mating	Litter	Results
R-22	N♀ × N♂	4	4 normal eyes
R-23	N♀ × N♂	3	3 normal eyes
R-24	N♀ × N♂	4	4 normal eyes
R-25	N♀ × N♂	5*	Partly absorbed
R-26	N♀ × N♂	6*	Partly absorbed
5		11–11*	11 normal eyes

* Degenerated.
N—Mouse having normal eyes.

ophthalmic, and three have normal eyes. These mice are being inbred and the incidence of unilateral anophthalmia will be studied.

DISCUSSION

Trypan blue is the first teratogenic agent to be tested in this study. The toxic effects of this dye have been reported by Gillman et al.[8–10] in rats. The mode of action, according to these authors, is that the erythrophagocytosis occurring soon after the injection is given may lead to an acute anemia accompanied by a decreased oxygen supply to the maternal tissues. Hamburgh[11] found that penetration of the dye particles is barred from passing through the placenta as well as through the yolk sac. Microscopic studies of our animals revealed the dye particles to be concentrated in the placenta and in the epithelium of the chorion but no gross or microscopic evidence of the dye was found in any of the embryos. We assume, therefore, that some degree of maternal hypoxia results from accumulation of the dye in the maternal tissues; but it is possible that some element of the dye, as yet unknown, may pass through the barriers and act directly upon the embryo.

We regard the production of craniorachischisis which results from extensive damage to the skull and brain of the embryo as simply a consequence of excessive dosage. Any abnormality of the eye present with this condition could be secondary to the initial

Fig. 3 (Barber). Uteri showing fetuses in various stages of absorption.

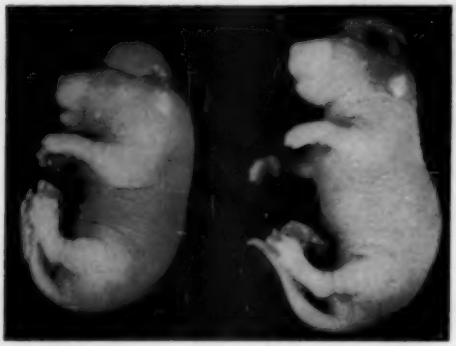

Fig. 4 (Barber). Two fetuses showing craniorachischisis.

Fig. 6 (Barber). Young female mice produced by N-61 (table 2) after treatment with 0.3-percent trypan blue. The animal on the left was backbred twice to the normal strain and produced only normal young. When back-bred to the anophthalmic strain, she produced young with normal eyes and bilaterally and unilaterally anophthalmic young.

effect to the neural folds. The strength of the trypan blue was reduced in our third experiment and was followed by a decrease in the incidence of craniorachischisis (table 2) (figs. 7 and 8).

The embryology of the anophthalmic mice used in these experiments was described by Chase and Chase[12] as an inhibition of the growth of the optic vesicles at 10 days or earlier. This retardation prevents the optic vesicles from forming a cup, or, if the cup forms, it is small and irregular and too far from the surface to induce a lens. Microscopic examination may reveal a few fragments of retinal tissue but a functional eye never develops.

Chase[13] ascribed this type of bilateral anophthalmia to the presence of two major recessive genetic factors, ey-1 and ey-2. Normal eyes occur in the presence of either Ey-1 or Ey-2. A reciprocal cross between the anophthalmic strain and a normal strain of mice would, therefore, produce a heterozygote having the alleles Ey-1 ey-1, Ey-2 ey-2, and possess normal eyes. This is the genetic condition of our untreated F_1 generation.

The F_1 generation produced from reciprocal crosses between the anophthalmic and normal mice always have normal eyes and 16 to 18 generations of brother-sister matings of their progeny are required to return about 90-percent anophthalmia to the strain. The results of our experiments (table 2) show that about one-third of the heterozygous F_1 generation were unilaterally anophthalmic when their mothers received injections of a 0.3-percent solution of trypan

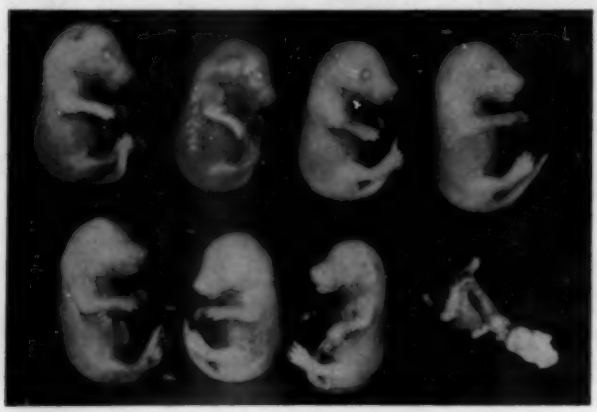

Fig. 5 (Barber). Young from Z-169, an anophthalmic female cross-bred with a male having normal eyes and given 0.25-cc. injections of trypan blue once daily on the seventh, eighth, and ninth day of gestation. There are four with normal eyes, one with craniorachischisis, one with right eye missing, and one partly absorbed.

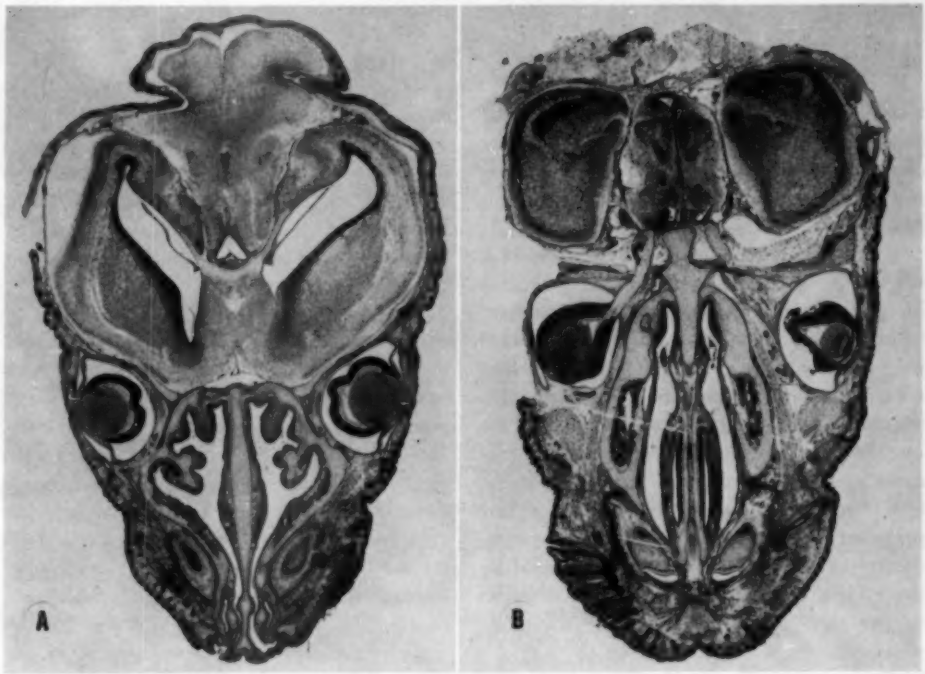

Fig. 7 (Barber). (A) Section through the head of a fetal mouse showing a bulge of the cranium and brain. (B) Section through the head of fetal mouse showing marked degeneration of the ectatic portion of the brain. The abnormality of the brain and skull has not prevented the formation of the eyes.

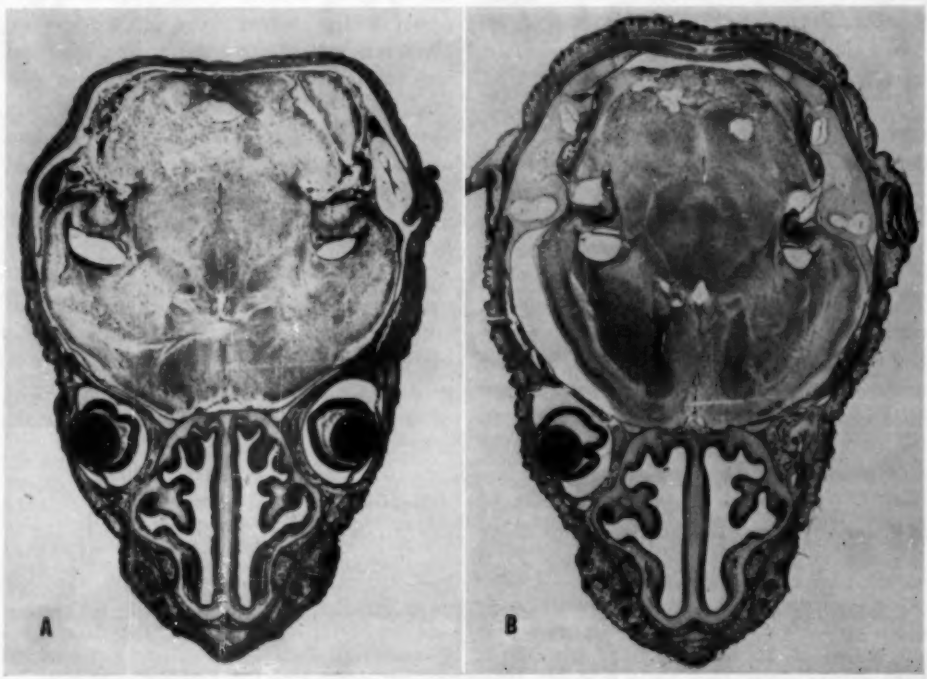

Fig. 8 (Barber). (A) Section through the head of a fetal mouse showing normal development of the eyes. (B) Section through the head of a litter mate showing the absence of one eye. Small fragments of pigmented epithelium are present in the orbit.

blue. Thus a congenital defect, similar to the condition carried as a recessive trait by one parent, was expressed in the first generation when the mothers were treated with a teratogenic agent.

It is difficult to explain the absence of a single eye when the genetic defect consists in the absence of both eyes. However, Chase,[13] who has studied this strain of mice very closely, feels that duplicate factors control the eyeless condition. It is possible that part of the control may also govern the uterine environment and the metabolic processes going on during the formation of the eye. Thus injections of trypan blue in the pregnant female may create an environmental situation similar to that produced by the eyeless gene, or it may act as a stimulus to the same disturbance of the metabolic processes.

An increase in the incidence of abnormalities inherent in certain strains of animals after the use of teratogenic agents during pregnancy has been shown by other investigators. Fraser et al.[14] found a marked increase in the incidence of cleft palate in mice following injections of cortisone; and O'Dell[15] increased the incidence of hydrocephalus in rats by the use of nutritional deficiences.

Eye defects, including anophthalmia, have been described following the use of other teratogenic agents as well as trypan blue[7, 10] in apparently normal strains of animals. These defects are similar to congenital abnormalities occurring in untreated animals and constitute phenocopies.[16, 17] Whether the unilateral anophthalmia that developed in our mice is a phenocopy or whether it is due to the creation of an environment comparable to that controlled by a genetic factor is impossible to determine at this time. However, if it could be shown that an abnormal uterine environment, created by drugs, nutritional deficiences, hormones, or disease processes,[18] does affect the expression of a recessive genetic trait, it might help to explain some instances of obscure or discontinuous inheritance in the human population.

Summary

Mice having recessive genetic factors for bilateral anophthalmia were mated with mice having dominant factors for eyes. The pregnant females were given 0.25 cc. subcutaneous injections of trypan blue on the seventh, eighth, and ninth day of gestation; 1.0-percent, 0.5-percent, and 0.3-percent solutions of trypan blue dye were used. One-percent trypan blue resulted in abortion or absorption in all the females tested. The incidence of abortion and absorption was less following injections of 0.5-percent trypan blue but there were many instances of brain and skull deformities. A few mice were apparently normal except for the absence of one eye. The effects of 0.3-percent trypan blue were less severe and permitted many feti to develop, about one half of these had normal eyes and slightly less had unilateral anophthalmia. One unilaterally anophthalmic female pup survived. When she was back-bred to the normal line, she produced young with normal eyes; but when back-bred to the anophthalmic line, she produced unilaterally and bilaterally anophthalmic young.

The suggestion is offered that a teratogenic agent may create an environmental stimulus or metabolic disturbance in the uterus comparable to that controlled by a genetic complex and result in the expression of a recessive congenital trait that would otherwise be suppressed.

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Discussion

Dr. Harold F. Falls (Ann Arbor, Michigan): Dr. Barber is to be highly commended for this well-controlled and well-presented study. She has selected, in trypan blue, an excellent teratogenic agent which it appears, to quote the author, "creates an environmental stimulus or metabolic disturbance in the intrauterine environment comparable to that controlled by a genetic complex and results in the expression of a congenital trait that would otherwise be suppressed." This study is most timely and is indeed welcome to our discipline. This is particularly so since T. H. Ingalls' writings have recently popularized the concept that maternal stress (specifically anoxia) during pregnancy is a very particular cause of congenital anomalies, a fact which he feels tends to shift the emphasis away from a genetic cause for many such anomalies. Many workers do not agree with his deductions.

I wish to digress at this time to emphasize that, while it has been believed that a considerable amount of congenital developmental pathology is gene ascertained, there is much evidence that environmental factors may modify a specific gene action. A particular gene action may be modified by at least three known factors: (1) the genotype in which it exists, (2) environmental agents, and (3) the genetic constitution and physiologic phenotype of the mother. To me, at least, it is important to note that these various agents apparently are not all acting by the same mechanism. The vast majority of congenital developmental defects are the resultant of complicated interaction of genetic and environmental factors.

Dr. Barber has guardedly assumed that unilateral anophthalmos is an expression of the action of the teratogenic agent which may create an environmental situation similar to that produced by the "eyeless gene" or "may act as a stimulus to the same disturbance of the metabolic process." This assumption is dangerous, at least to me, in light of our present level of knowledge concerning the chemical processes occurring during organogenesis.

Fern and Wilson have both expressed the belief that trypan blue acts directly upon fetal tissue. Hamburgh feels that this agent has a direct teratogenic effect on the nervous and circulatory system of the rat embryo. Failure of closure of the neural tube and eyelessness occur in his fetuses.

Gilman and co-workers found frequent unilateral anophthalmos in their fetuses and particularly noteworthy was the frequency of diencephalon injury. Their eye defects ranged from mild unilateral or bilateral microphthalmos to total absence of the eye. The central nervous system was particularly deleteriously influenced by the agent.

In certain species it thus appears that unilateral

anophthalmos may result from the direct action of trypan blue regardless of the genetic make-up of the fetuses. It is impossible, therefore, to state, on the basis of the data presented, whether Dr. Barber's fetuses exhibit unilateral anophthalmos as a phenocopy or actually exhibit the effect of an environment permitting expression of a heterozygous gene. I favor the former possibility (phenocopy), particularly since her homozygous recessive stock did not exhibit "craniorachischisis" or unilateral anophthalmos. It might be profitable for Dr. Barber to terminate the pregnancies at successively early stages to ascertain what is occurring in the absorbed and partially absorbed fetuses.

Dr. Barber has intimated hypoxia as being the effect of the trypan blue and in turn the cause of the unilateral anophthalmos. Since this (anoxia) is doubted by some workers, it might be profitable to repeat the present study and employ anoxia as the specific and only agent.

I also urge, as the author has done, caution in applying such laboratory findings to explain human pathology.

It has been a sincere pleasure to discuss this paper and I am additionally grateful to Dr. Barber for previously permitting me to review her paper.

DISTRIBUTION OF RADIOACTIVE SULFATE IN THE DEVELOPING EYE*

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The development of the connective-tissue structures of the eye has been well described in man and some animals, but always with reference to their collagenous component. Although these form the most obvious portion, the cells and the ground substance are perhaps more important physiologically.

Ground substance has been investigated histochemically and by means of radioautography in the adult eye, in which S^{35} labeled mucopolysaccharides have been demonstrated. Metachromatic staining of the cornea has been reported in two well-developed rabbit fetuses,[1] and sulfate labeled with S^{35} was observed by Bostrom and Odeblad[2] in the sclera of a three-week-old rabbit embryo.

Excepting in a preliminary report,[3] no study has been made of the ground substance at different stages of the developing eye. It is a necessary element in fibrillogenesis, and the cornea may owe its transparency in part to its high mucopolysaccharide content. The vitreous humor is in reality a type of connective tissue in which the fibrous and cellular elements are reduced in quantity, and in which the mucopolysaccharide content is the predominant feature. It is of extreme importance in the embryo because through it the vascular supply reaches the internal structures, and the growth of the eye as a whole depends on distention of the retina and sclera by the tissue in the optic cup, that is, vitreous humor (Coulombre[4]). The ground substance of the ocular connective tissue is of obvious importance in both embryo and adult and its development was, therefore, studied.

METHODS

New Zealand white rabbits, about six pounds in weight, were bred to obtain embryos of known age. Stages from the 14th day of gestation through term, and the postnatal period from the first to the 20th day, were studied. The pregnant rabbits were anesthetized with Nembutal and the embryos removed and placed in appropriate fixatives immediately.

Synthesis of the sulfated mucopolysaccharides can conveniently be demonstrated by the use of S^{35} sulfate, which in connective

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tissues is incorporated into mucopolysaccharides of the ground substance, such as chondroitin sulfate. The site at which the sulfated materials form can be determined in radioautographs. One mc. per kg. S^{35} sulfate was injected intravenously seven hours before autopsy. Therefore, sulfated mucopolysaccharides synthesized during this period were labeled.

Embryos were fixed in Carnoy's solution and autographs prepared by coating with bulk emulsions, Ansco A or NTB_2, according to the method of Gross,[5] or by the wet mounting technique (Boyd[6]), using Eastman autoradiographic or NTB_2 plates. The reduction of silver particles in the photographic emulsion indicates the presence of organically bound sulfate in the adjacent tissue. Only sulfate bound in organic complexes are shown, because very little inorganic sulfate remains in the body seven hours after an intravenous injection, and any remaining trace would be washed out during preparation of the sections prior to staining.

Bostrom and Mansson[7] have shown that S^{35} labeled inorganic sulfate is incorporated almost exclusively in the mucopolysaccharide component of connective tissue. Study of such autographs of embryos of various ages makes it possible to determine the time when the embryo first forms sulfated connective-tissue ground substances, as well as the approximate amounts and location where this occurs.

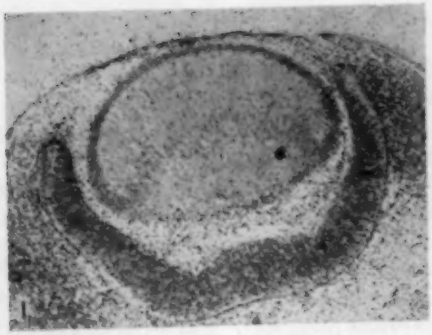
Reticular and collagenous fibers were stained with Mallory's trichrome or Wilder's silver technique after formalin fixation. The metachromatic reaction with toluidine blue, which demonstrates the presence of mucopolysaccharides, both sulfated and nonsulfated types, was employed according to the method of Lison on tissues fixed in a lead acetate formol mixture. The periodic acid Schiff (PAS) technique was used (on eyes fixed by freeze substitution (Simpson[8]) to demonstrate the carbohydrate associated with the fibers (Glegg[9]).

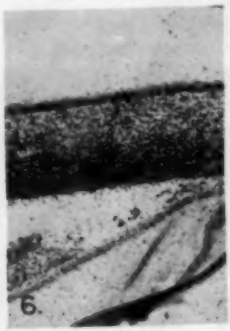
Results

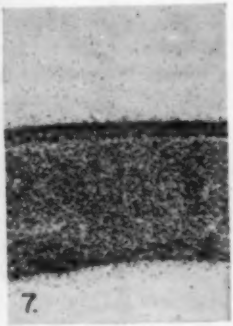
The eye of the rabbit of the 14th day of gestation was well formed, with a solid lens of primary fibers and a retinal cup filled with vascular vitreous humor, but the connective tissue coats had not yet developed. Mesenchymal cells were migrating into the eye to form Descemet's endothelium and the corneal stroma. The corneal cells were loosely arranged, irregularly distributed, and underlying a simple corneal epithelium. No connective-tissue fibers were demonstrable, and the region of the future cornea and sclera stained very lightly with periodic acid-Schiff. None of the mesenshymal connective tissue was metachromatic. However, incorporation of S^{35}, presumably in a connective-tissue ground substance, could be demonstrated. This appeared to be restricted to a narrow zone at the interface between the vitreous and the retina or lens (fig. 1). The center of the vitreous humor was filled with blood vessels which showed, together with the lens, no accumulation of sulfate. The amount of corneal stroma in the center region was too little to be certain that S^{35} was present, but there was an indication that sulfate was being laid down in the limbal zone.

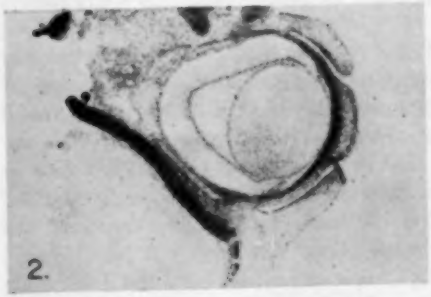
The changes from the 14th to the 17th day were marked; the corneal stroma increased in amount and became slightly more dense, and an argyrophilic basement membrane formed in its center just under the epithelium. This was more strongly developed by the 18th day. The metachromasia of such corneas was confined to the posterior layers and was not seen in relation to the basement membrane. The material responsible for the metachromasia was continuous laterally into the anterior portions of the future sclera, and was extremely sensitive to testicular hyaluronidase. The basement membrane could be stained by the PAS technique, but the posterior corneal layers were still exceedingly faint.

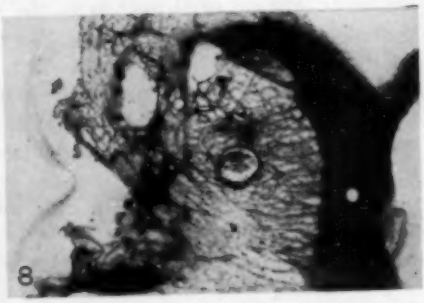
Radioautographs of embryos of this stage showed a strong sulfate accumulation throughout the cornea, much greater in

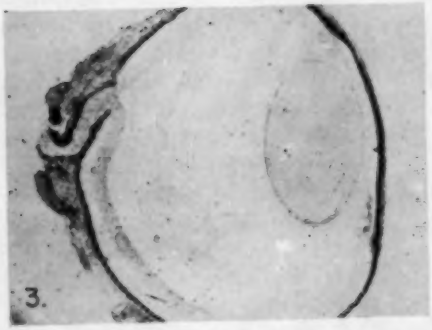


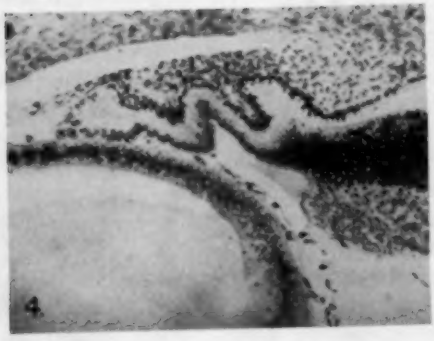












amount in the deeper metachromatic layers (fig. 2). Sulfated material was also demonstrable in the most anterior part of the sclera. At this stage, collagen fibers stained with aniline blue were easily seen in the posterior corneal layers where they were continuous with fibers of the sclera forming in the limbal area.

The most striking feature of radioautographs of eyes at this stage was an accumulation of sulfate on or in the vitreous surface adjacent to the retina and lens. The resolution of the autographs was not sufficient to determine whether the sulfate was deposited solely within the vitreous, or also within the internal limiting membrane of the retina. Although a network of hyaloid vessels was very close to the surface of the vitreous, it did not seem likely that the sulfate was associated with them, for the interior of the vitreous humor was also filled with a branching network of blood vessels and cells which appeared negative in the radioautographs.

Embryos were studied in a closely graded series from the 18th to the 26th day, by which time the connective tissue capsule was well formed completely around the eye. Collagenous fibers were distributed throughout the cornea, but were regularly arranged and dense in the posterior layers and very loose and irregular in the anterior half. The posterior corneal connective tissue was continuous with that of the sclera which, by this stage, completely enveloped the eye to the optic nerve, which in turn, had developed its own connective-tissue sheath (fig. 3). Both the cornea and the sclera were extremely metachromatic. Although the sclera in the adult is not metachromatic, this type of staining reaction is extremely marked in embryos of this age, exceeding that of the cornea. This may be due to a denser arrangement of the scleral fibers than those of the cornea. The corneal metachromasia was much more marked in the deeper layers than in the loose anterior ones. The connective-tissue fibers of both the cornea and the sclera were more mature at this stage, as shown by the deep-

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Figs. 1-9 (Smelser and Ozanics). Distribution of radioactive sulfate in the developing eye.

(1) Radioautograph of a section of an eye of a 14-day-old rabbit embryo showing accumulation of S^{35} in cornea and at interface of the vitreous and retina and lens. The silver grains, reduced by the action of S^{35} are shown superimposed on the section which was stained with hematoxylin.

(2) Radioautograph of a section of the eye of an 18-day-old rabbit embryo. Note concentration of S^{35} in the posterior layers of the cornea and the strong reaction at the interface of the vitreous humor and the adjacent retina and lens. The dense black structure above the eye is the cartilage and bone forming the roof of the orbit. The lesser density of the central corneal layers is due to distortion of those lamallae during preparation. The section itself is lightly stained.

(3) Radioautograph of the eye of a 26-day-old rabbit embryo. S^{35} has been incorporated into the sclera completely around the eye and in the optic nerve sheath, as well as in the cornea. The concentration seen earlier on the surface of the vitreous humor has disappeared by this stage. The section is stained with hematoxylin.

(4) Section of the region of the ora serrata of a 22-day-old rabbit embryo showing a stainable (hematoxylin) coagulum of vitreous humor on the surface of the ciliary body adjacent to the retina.

(5) Radioautograph of a section of the same eye as that shown in Figure 4. Note the concentration of S^{35} in the vitreous humor, in the region of the coagulum shown in Figure 4, and in contact with the ciliary body. The section is stained with hematoxylin so that the structures of this region are shown in addition to location of S^{35}.

(6) Radioautograph of the cornea of a rabbit five days post partum. Note S^{35}-labeled sulfate is still more concentrated in the posterior layers.

(7) Radioautograph of the cornea of a rabbit 18 days post partum. The S^{35}-labeled sulfate is now homogeneously distributed throughout the cornea.

(8) Radioautograph of a whole mount of the vitreous humor and ciliary body of a 22-day-old rabbit embryo. Most of the vitreous humor shows no evidence of sulfate incorporation, but the ciliary body contains much S^{35} in it or on its surface as indicated by the marked reduction of silver grains in the emulsion covering it.

(9) Radioautograph of a section of the eye of a 22-day-old embryo. The vitreous is shrunken and detached from the retina. In pulling away some of the S^{35}-labeled material remained with the retina and some was part of the vitreous humor.

ening color obtained with the periodic acid-Schiff technique although, relative to the adult tissue, the staining was still very weak.

On the 26th day, the still quite vascular vitreous humor showed little or no sulfate incorporation in relation either to the vessels or to the interface of the vitreous and retina. However, at about this stage, the ciliary body and processes were well differentiated and on their inner surface, particularly near the ora serrata, a stainable coagulum of vitreous humor was often found. This material gave a strong metachromatic reaction with toluidine blue which was abolished with hyaluronidase, and radioautographs of this region showed an accumulation of sulfate in this material. This is shown in a 22-day embryo (figs. 4 and 5). The only sulfated substance found in the vitreous humor was in that region where it was still in contact with the ciliary processes, near the ora serrata, and adjacent to the future pars plana. No visible zonular fibers had developed by this stage.

At term, the differentiation of the choroid was easily demonstrable. It consisted of an extremely vascular and loose connective tissue, the majority of the fibers of which were reticular, supporting the blood vessels and separating the sclera from the pigment epithelial layer of the retina.

At birth and during the first few days of postnatal life, the cornea was still obviously immature. The posterior layers were dense and regular, whereas the anterior layers were of loose, irregular collagenous fibers. The intensity of the metachromasia was greatly increased, but still far less than that found in the adult. The sclera was still markedly metachromatic, approximately equal in intensity to that of the cornea. The metachromasia of the cornea was no longer completely abolished by pretreatment with hyaluronidase, whereas that of the sclera was wholly sensitive to this treatment.

Radioautographs showed a distribution of sulfate very similar to that of the metachromatic material. Although sulfated mucopolysaccharides were being formed throughout the cornea, their rate of synthesis was still greater in the posterior layers (fig. 6). The sclera also was synthesizing this type of material at a rate not significantly different from that of the cornea. Marked changes in the cornea and sclera did not occur during the first two weeks after birth, excepting that the metachromasia of the sclera faded perceptibly in the posterior hemisphere.

By the 19th to 20th day, or approximately one week after the lid adhesions had broken down, the eye appeared to be essentially mature. The cornea was transparent and, for the first time, the corneal metachromasia was equal in intensity in the anterior and posterior layers, and like that of the adult. The metachromasia of the sclera had been lost except in the limbal areas. Sulfate uptake by the corneal tissue, as shown in autographs, was essentially equal in the anterior and the posterior layers (fig. 7) as in the adult, but the rate of sulfated mucopolysaccharide synthesis was still very high.

Vitreous humor

The vitreous humor of the adult contains considerable quantities of hyaluronic acid which is metachromatic, but sections of the embryonic vitreous showed no metachromasia excepting near the ora serrata, as already mentioned. Even in the early stages before a well-developed ciliary body formed, metachromatic vitreous was found lying on the inner edge of the lips of the optic cup. It was thought that the absence of metachromasia throughout the vitreous might be due to its low concentration and the thinness of the sections. Therefore, the eyes of a series of embryos were dissected and the whole vitreous spread on a slide with some fragments of attached retina and ciliary body. These were dried, thus concentrating all of the metachromatic substance in the entire vitreous into a thin film.

The vascular network was a prominent feature of these preparations, and stained orthochromatically as did the isolated cells

which were scattered through it. The retina and the ciliary body both stained orthochromatically, but the material adjacent to the ciliary body was extremely metachromatic.

Since, in the late stages of development, concentration of sulfate was found associated with the ciliary body, particularly at the ora serrata, spreads of vitreous humor of embryos treated with S^{35} sulfate were also made and autographs prepared of them. No concentration of sulfate was found associated with the vitreous humor cells and very little with the vascular network. Sulfate was, however, found irregularly distributed in the vitreous coagulum, particularly against the ciliary body, and occasionally near fragments of retina which had been included in the spreads (fig. 8).

Thus, both the preparations stained with toluidine blue and the radioautographs suggest that the materials demonstrated by these methods originate or become concentrated on the surface of the vitreous humor, particularly in relation to the ciliary region and the internal limiting membrane of the retina, rather than in relation to the vascular sytem and its attendant cells.

Discussion

Descriptions of the developing eye (Bach and Seefelder,[10] and Ida Mann[11]) have dealt with the distribution of the collagenous fibers of the embryonic cornea and sclera. These have been shown to arise first in the deep or posterior layers of the cornea, whence they spread to the anterior or limbal portion of the sclera. Scleral organization proceeds rapidly to the posterior pole where the fibers encircle those of the optic nerve. The sclera then thickens as the eye grows, and the corneal fibers develop in its anterior layers.

The present study finds that, in general, development of metachromatic and sulfated materials of the ground substance follows the same pattern, but precedes fiber formation by a short interval of time. An exception was found in that the first connective-tissue fibers formed were not posterior corneal collagen, but were anterior argyrophilic fibers forming the basement membrane under the epithelium. Their origin was either not associated with metachromatic or sulfated precursors, or these substances were present in such small amounts and arranged in such thin lines that they were not readily visible.

Reticular fibers elsewhere, for example, regenerating wounds[12] and basement membrane of the lid epithelium, were associated with sulfated and metachromatic ground substance. The mucopolysaccharides which the methods used here located, were associated predominantly with collagen formation in the developing eye. The cornea reached an adult-like structure late in development.

When the eyes first opened at about the 12th postnatal day, fiber formation was incomplete in the anterior layers, and the degree of metachromasia was not yet equal to that of the adult cornea, nor had the normal degree of transparency or hydration been obtained.[13] Apparently, complete homogeneous corneal sulfation and adult degree of metachromasia, hydration, and transparency were all reached simultaneously late in development.

During differentiation, collagenous connective tissues in general are highly metachromatic, whereas in the adult most of them are not. Sclera is a typical example. Its high metachromasia during early development was lost rather rapidly in the late stages. However, the cornea did not follow this pattern. It increased in metachromasia as it progressed to full structural maturity. During embryonic development the metachromatic substances were readily removed by treatment with testicular hyaluronidase, whereas the material formed in later stages of corneal development was increasingly resistant to this enzyme. This indicates that these last formed metachromatic substances were not hyaluronic acid, chondroitin, or chondroitin sulfate A or C. Possibly keratosulfate was formed at this stage.

It was not possible to find traces of metachromasia at stages earlier than those showing incorporation of sulfate in the tissues. One might expect hyaluronic acid and chondroitin to be formed first and the latter to be subsequently sulfated. If this does occur, the quantities must be so small and/or the nonsulfated stage passed so quickly that evidence of their presence could not be obtained by the metachromatic color reaction.

Although the vitreous humor contains much hyaluronic acid, its source is unknown. In the embyro, Bembridge and Pirie[14] were unable to find evidence of its presence—a somewhat surprising report because one might expect to find a high concentration of mucopolysaccharides in growing connective tissue. Although metachromatic substances were readily demonstrable in the embryonic vitreous humor, the specific type of mucopolysaccharide present could not be established by this means. The concentration of metachromatic material found in both sections and whole mounts, near the ciliary body at the ora serrata in the embryo, suggests that it is produced in this region, rather than by the cells of the vitreous humor, which are associated with the hyaloid vascular network.

In view of the well-established fact that the adult vitreous humor does not contain sulfated mucopolysaccharides,[15] their presence in the developing vitreous was surprising. The structure of the embryonic vitreous is, however, markedly different from that of the adult, the bulk of which forms after the sulfated stage has been passed. The sulfated materials were not associated with the cellular or vascular components, but with the vitreous surface, which, at this stage, is firmly attached to the retina posteriorly. It was, therefore, impossible to determine the origin of the sulfate in this area.

Figure 9 shows a specimen in which the vitreous body is shrunken away from the retina. Some of the sulfated material remained with the vitreous and the attached retinal remnant, and some with the retina. The vascular surface of the vitreous which was applied to the posterior pole of the lens was also rich in sulfated materials. It is possible that this portion is largely derived from the ciliary region and diffuses under the lens and, like that in the posterior area, is derived from the retinal cup.

Summary

1. The first connective-tissue fibers forming about the developing lens and retinal cup are argyrophilic and form the corneal basement membrane. Collagenous fibers quickly develop in the posterior corneal layers and fibrillogenesis spreads around the eye to the posterior pole, forming the sclera.

2. The anterior lamellae of the cornea are the last ones to form and are completed postnatally.

3. Connective tissue ground substances, as indicated by metachromatic staining and S^{35} sulfate incorporation, precede the formation of collagenous fibers.

4. Whereas scleral metachromasia decreases with development, that of the cornea increases in degree and in resistance to hyaluronidase. This suggests that keratosulfate, which is characteristic of the cornea and is not split by hyaluronidase, is first formed late in development.

5. Sulfated connective-tissue components, presumably mucopolysaccharides, can be demonstrated in radioautographs very early in development. Apparently the nonsulfated mucopolysaccharides such as chondroitin and hyaluronic acid are not present in appreciable quantities earlier than those which are sulfated.

6. The first clear traces of sulfate incorporation in the embyronic eye occur in or on the surface of the vitreous humor. The sulfated component is not associated with the hyaloid vessels or with the cells found in the vitreous, but is found in close association with the vitreous humor surface in contact with the internal limiting membrane of the retina, the anterior hyaloid membrane, and the surface of the ciliary body.

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Discussion

Dr. Ludwig von Sallmann (Bethesda, Maryland): I am afraid I don't have much to say in discussion, because I have in no way participated in this work of Smelser and Ozanics. My only contact with the problem was many years ago when I tried the use of radioactive sulfate in early autoradiographic studies on the eye, which have not been followed up with respect to the distribution of radio sulfur. Dr. Smelser and Miss Ozanics approached the development problem most successfully with a technique which provided new and important information. From the technical viewpoint I have never seen finer autoradiographs of a whole eye.

I might ask Dr. Smelser two questions. In the second slide the area in the film corresponding to the vitreous appears unevenly blackened. Is this an artefact, or is some significance attached to this difference in density?

Second, how can one be sure of having removed the unbound inorganic radioactive sulfate completely and left intact the sulfonated mucopolysaccharide?

Dr. Norman Ashton (London): I should like to congratulate Dr. Smelser on this magnificent work.

We found, some time ago, that the connective-tissue elements enter the retina with the ingrowth of the blood vessels. One can see aggregations of mesenchymal cells preceding the ingrowth vessels, and we found that they contained what apparently were glycogen granules.

I should be most interested to know whether Dr. Smelser has found any radioactive sulfate preceding the ingrowth of vessels into the retina.

Dr. George K. Smelser (closing): I wish to express my appreciation of the discussion and will try first to answer Dr. von Sallmann's question relative to Figure 9.

The technique used is that of coating and there is often some degree of unevenness in the thickness of the emulsion between one side of the slide and the other. Usually in a small area this is of no significance. In the case of the particular section to which you refer there was a marked artefact, due to shrinkage of the vitreous which allowed us to demonstrate, I think, that the sulfated mucopolysaccharide was attached rather firmly to the vitreous as well as to the retinal surface. On the opposite side of the section where the autograph was lighter, the emulsion was thinner and there were fewer grains to be photographed.

One can be fairly certain that the autography is of organically bound sulfate and not of free sulfate. I said it was sulfated mucopolysaccharide because it has been shown by a number of very capable chemists that inorganic sulfate, injected intravenously, can be found in the connective tissue, and

from this connective tissue S^{35}-labeled chondroitin sulfate of various sorts can be isolated. All of the sulfate can be found in those compounds, in connective tissue. In addition, seven hours after an intravenous injection of inorganic sulfate, most of it has been excreted by the kidneys and very little left in the blood. Traces left in the free fluid in the connective tissue spaces would be removed because after the sections are prepared they are stained and washed in water, thus washing out unbound sulfate. One does not know the exact form of the S^{35} in our sections, but we are dependent on the excellent work of Boström, his co-workers, and of other laboratories in stating that the binding here is in the form of chondroitin sulfate and, we feel sure, keratosulfate also.

In answer to Dr. Ashton's question relative to the ingrowth of blood vessels, I would rather expect that if there is much mesenchymal material entering the retina with the blood vessels, one would find either simultaneously or preceding them, the ingrowth of material which was sulfated, because one generally finds chondroitin sulfate in the ground substance of such tissue.

We have not followed the blood vessel growth in the embryonic retina by this method. Some of the preparations which we have now shown you do show that there is a condensation of this sulfated material in the layers around blood vessels in the retina. The resolution of this type of radioautograph is not quite adequate to determine if the S^{35} is within the connective tissue surrounding a small capillary or not. One can only say it is in the neighborhood of it.

By the use of a stripping film technique which has been developed so very excellently by Pelc, one could be certain, I think, that the sulfate would be in the adventitia of small vessels. If the adventitia is rather limited, one will be in difficulty trying to demonstrate sulfate in ingrowing vessels.

OXYGEN STUDIES IN RETROLENTAL FIBROPLASIA*

VI. The effect of concentration and duration of exposure to oxygen on the immature mouse eye

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Previous clinical and experimental studies[1-7] have convincingly demonstrated that the overuse of oxygen is an important and apparently the principal factor in the etiology of retrolental fibroplasia. In animal experiments the severity and incidence of ocular lesions produced by oxygen in general have been found to be proportional to the concentration of oxygen used and the duration of exposure to oxygen. Only those animals with an incomplete retinal vasculature show ocular lesions on exposure to oxygen and the susceptibility to oxygen damage is inversely proportional to the degree of vascularization of the immature retina.[6] The present experiments were designed to observe more quantitatively the effects of duration of exposure of the animal to oxygen and the influence of smaller differences of oxygen concentration on oxygen-induced lesions in the mouse eye.

Subjects and Methods

All animals used in this study were newborn albino from the general purpose colony (National Institutes of Health). Nursing mother animals with their newborn litters were placed in oxygen chambers within the first 18 hours of birth.

The oxygen chambers consisted of the plexiglass tops of the Isolette incubator which were furnished us by the Air-Shields Co. The air-conditioning and humidification units of the regular Isolette were not included in these units. As a substitute to insure circulation of the gases and an even concentration of oxygen throughout the

* From the Wilmer Ophthalmological Institute of The Johns Hopkins Hospital and University, Baltimore, and the Retrolental Fibroplasia Research Laboratory, District of Columbia General Hospital, Washington, D.C. These studies were aided by grants from the E. Matilda Ziegler Foundation for the Blind, the National Society for the Prevention of Blindness, and the National Institute of Neurological Diseases and Blindness, U. S. Public Health Service.

chamber, a small two-inch circulating fan was mounted at one end of the chamber. A thermostatically controlled heating coil lined the bottom of the chamber and the temperature could be regulated as desired.

During these experiments the oxygen concentration was measured at intervals varying between four and 12 hours on a regular basis. A paramagnetic analyzer, accurate within ±2.0-percent concentration, was used to measure the oxygen content. No samplings of CO_2 were made during these studies, as sufficient data showing no significant CO_2 accumulation had been collected in previous experiments under similar circumstances.

Litter mates of the same strain of animals raised in room air served as controls for a part of these experiments. Stock controls from previous experiments on the same strain of mice were also included. Where concentrations of 80-percent oxygen or more were used, a system of exchanging nursing mother animals with foster mothers maintained in room air was required to prevent pulmonary irritation of oxygen on the mother animals. At 24-hour intervals the mother animals were removed from the chamber and swapped with their counterparts in room air. The newborn animals, however, withstood the high oxygen concentration for much longer periods before any evidence of pulmonary difficulty appeared. Where concentrations of oxygen of 60 percent or less were utilized, it was unnecessary to remove the mother animals from oxygen as they tolerated the oxygen without evidence of pulmonary irritation.

The animals were kept in the incubators for periods varying from one to 10 days. After the desired stay in oxygen was completed, the animals were transferred directly to cages in room air and held until killing. This was usually done at about the 18th day of age. Some animals that were raised in 30-percent and 40-percent oxygen environment were continued at these concentrations in the incubator for either 14 or 21 days (table 1).

At the conclusion of the experiments the animals were anesthetized with ether anesthesia and the chest wall opened. An intracardiac injection of India ink diluted 50 percent with distilled water was injected slowly into the beating animal heart. Care was taken to avoid excess pressure during injection into the heart. The pumping action of the heart circulated the India ink throughout the body and when the animal's tongue appeared black, the injection was stopped. Usually the heart stopped beating within a few seconds after this. The eyes were

TABLE 1

SHOWING EFFECTS OF VARIOUS CONCENTRATIONS OF OXYGEN FOR 10 DAYS TO THE NEWBORN MOUSE

Oxygen Concentration (percent)	No. of Animals	No. with Ocular Lesions
20 (room air)	96	0
30	27	0
40	40	4
60	16	16
80	18	18

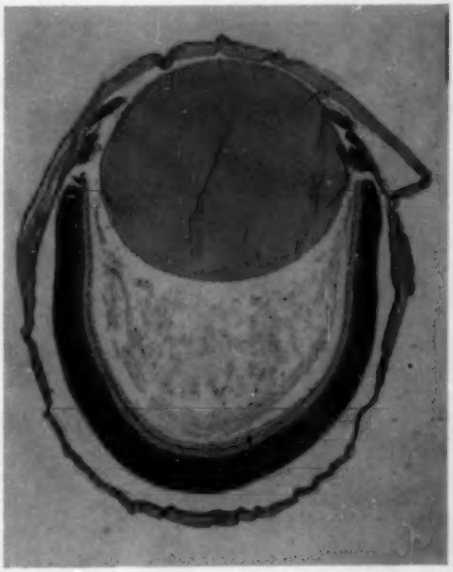

Fig. 1 (Patz and Eastham). Cross-section of newborn mouse eye, showing hyaloid vessels in the vitreous, vessels of the tunica vasculosa lentis, and the undifferentiated pattern of retina. (Hematoxylin-eosin ×30.)

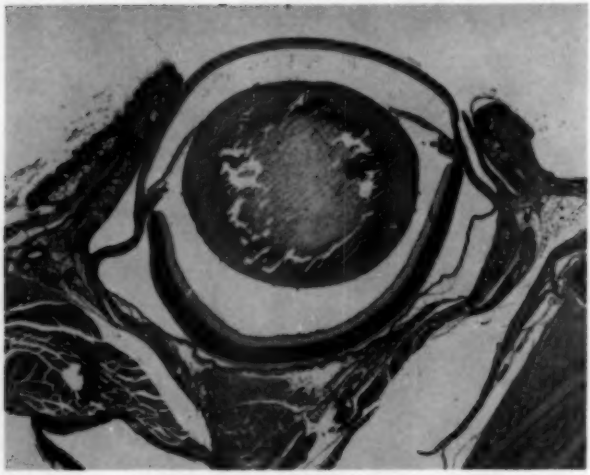

Fig. 2 (Patz and Eastham). Eye of 18-day-old control mouse, showing clear vitreous with complete regression of hyaloid vessels and tunica vasculosa lentis. Note that the retina has differentiated into its adult pattern. (Hematoxylin-eosin ×30.)

enucleated and fixed in Bouin's solution, then transferred after 24 hours to formalin fixation. One eye of each animal was embedded in paraffin and sectioned in the usual manner in sections six microns in thickness. The opposite eye was opened at the limbus and the cornea, iris, and lens were removed. The retina was then dissected out and placed on a slide with the vitreous surface up. Glycerin jelly was applied and a cover slip placed over the glycerin-mounted specimen. The paraffin-embedded sections were stained with hematoxylin and eosin. The flat retina preparations which showed the India ink injected vascular system were studied unstained.

Results

The ocular lesions in the oxygen-treated animals consisted of nodules of endothelial cells in the nerve-fiber layer of the retina, capillaries erupting from the internal layers of the retina into the vitreous, intraocular hemorrhages, and abnormal persistence and

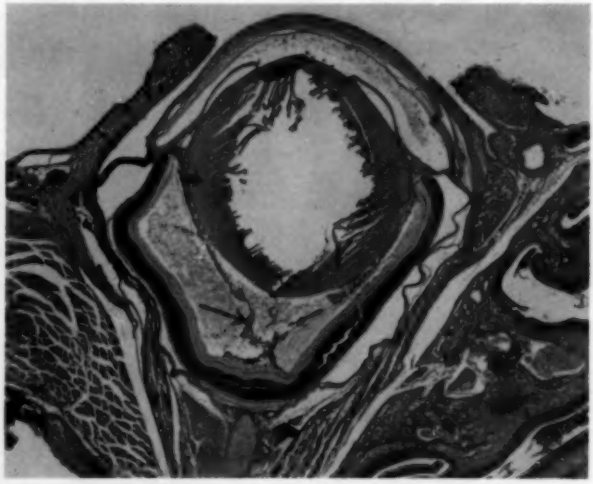

Fig. 3 (Patz and Eastham). An 18-day-old mouse who received 80-percent oxygen for five days and was removed to air for an additional 13 days. Note persistence and proliferation of hyaloid vessels and disorganization of the vitreous. (Hematoxylin-eosin, ×30.)

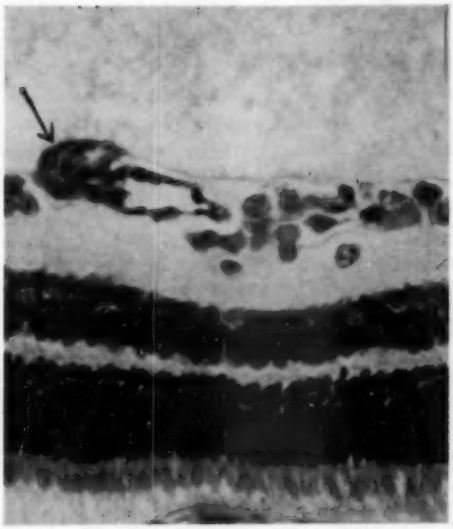

Fig. 4 (Patz and Eastham). Cross-section of retina of 18-day-old mouse who received 80-percent oxygen for five days and was placed in room air for 13 days. Arrow points to endothelial nodule proliferating from retina into the vitreous which is disorganized. (Hematoxylin-eosin, ×500.)

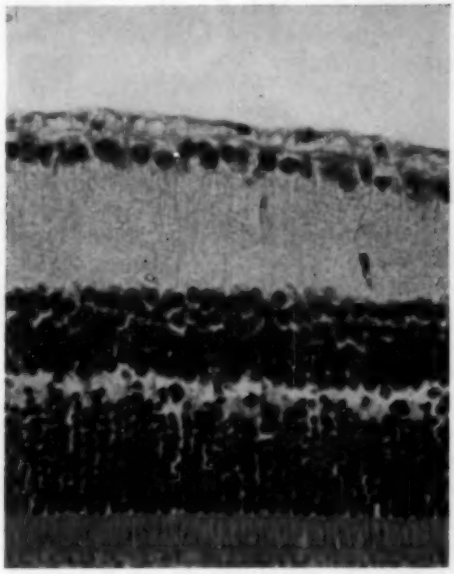

Fig. 5 (Patz and Eastham). Cross-section of normal 18-day-old mouse eye serving as control. (Hematoxylin-eosin, ×500.)

proliferation of the hyaloid vasculature (figs. 1 to 5). None of these changes was detected in any of the control animals.

The data show that animals exposed to 30-percent oxygen for a period as long as 21 days continuously showed no abnormalities visible in either flat retina preparations or in cross section.

At concentrations of 40-percent oxygen, no lesions were noted after exposures up to and including seven days. Where the stay in oxygen was extended to 10 days, four of 40 animals (10 percent) showed ocular hemorrhages. In this group there were no vascular proliferative lesions noted; however, intraocular hemorrhages occurred as compared with no hemorrhages in any of the controls. Where the stay in oxygen at 40 percent was increased to 14 days, five of 24 animals (20.8 percent) showed ocular lesions consisting of ocular hemorrhages and neovascular buds proliferating from the retina into the vitreous.

TABLE 2

SHOWING EFFECT OF DURATION OF EXPOSURE TO OXYGEN AT 40-PERCENT CONCENTRATION ON THE NEWBORN MOUSE EYE

Days Exposure to 40-percent Oxygen	Total No. of Animals	No. with Ocular Lesions
7	12	0
10	40	4
14	24	5

At 60-percent oxygen concentration, no lesions were noted during the first four days of exposure to oxygen. However, 10 percent of the animals showed lesions after five days' exposure and 100 percent of the animals showed ocular lesions after extending the exposure to seven days.

At 80-percent concentration, no lesions appeared in the first four days. After only one additional day of exposure to make a total of five days in oxygen, 60 percent of the animals showed ocular lesions. Extending the stay in oxygen at 80-percent concentration for an additional two days for a total

exposure of seven days resulted in 100 percent of the animals showing ocular lesions (tables 1 and 2).

Discussion

These data show very strikingly the importance of duration of exposure as a factor in experimentally induced lesions. It is noted that in general the greater the concentration of oxygen, the more frequent and earlier the ocular lesions appeared. The effects of duration of exposure were more striking, however. For example, the difference in exposure to 80-percent oxygen for four days and for five days was quite remarkable. No lesions occurred after four days' exposure and for one additional day to make a total of five days in oxygen, 60 percent of the animals showed ocular lesions.

We should be cautious here in applying directly these animal data to the problem of clinical retrolental fibroplasia. However, the experiments at 40-percent concentration have possible clinical application in light of some of the current recommendations on oxygen usage in the premature nursery. A feeling of security that 40-percent oxygen is a safe level for the premature infant has developed in certain premature centers. These data showing lesions occurring after 10 and 14 days' exposure at 40-percent oxygen are quite pertinent to this question. They should sound a note of caution against the *indiscriminate* use of so-called safe levels of oxygen (40 percent or under) for prolonged periods, as such a policy may not be without ocular danger in the premature nursery. Our previous data show that the kitten and puppy are more susceptible to oxygen induced ocular lesions than the mouse and it is possible that the premature infant may be even more susceptible.

It is of interest that during the last few days of prolonged exposure to oxygen the animal retina is more differentiated and theoretically we might think less susceptible to ocular damage from oxygen. These data, therefore, raise the possibility of a cumulative effect from the prolonged exposure to oxygen. It was of interest to note that the animals at 40-percent oxygen who were exposed for seven days showed a failure of the deeper vascular net of the retina to develop, although the retina otherwise appeared normal. This observation was first reported by Hellström[9] and is confirmed in these experiments.

These data which emphasize the apparent critical role of duration of exposure to oxygen are in general agreement with a similar experiment previously reported from our laboratory[8] in controlled experiments on the effect of rapid versus gradual withdrawal from oxygen. In these latter experiments it was noted that following an initial nontoxic exposure of the animal to oxygen, the added oxygen that was administered during a period of gradual tapering off (withdrawal) from oxygen apparently furnished that extra amount of oxygen needed to make the *total* exposure injurious to the animal eye.

It is important to compare these data with the excellent studies of Hellström[9] who used C-57 black mice. Hellström found 40-percent oxygen exposure injurious to approximately one third of his newborn animals after only five days' exposure and correspondingly slightly increased sensitivity to the higher oxygen concentrations than were found in the albino mice reported here. It seems reasonable that some difference in susceptibility of these strains may exist. However, slight differences in technique may account partly for these minor discrepancies.

Summary and Conclusions

1. Newborn mice were placed in oxygen at concentrations of from 30 to 80 percent for periods varying between one and 21 days.

2. It was noted that 30-percent oxygen had no injurious effect on the animal eye and the stay in 40-percent oxygen became harmful after exposure from between seven and 10 days.

3. These data stress the importance of duration of exposure to oxygen as an ex-

tremely important factor in determining the incidence and severity of lesions noted experimentally.

4. The possible clinical application of these data is briefly cited.

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Discussion

Dr. V. Everett Kinsey (Detroit): I would like to congratulate Dr. Patz on the excellent presentation of his interesting work on retrolental fibroplasia, and to emphasize his last few remarks with regard to the potential hazards of administering oxygen, albeit in concentrations of 40 percent or less.

For that purpose, may I present two slides of data obtained by the Co-operative Study on Retrolental Fibroplasia published last October.* The first shows the relation between the concentrations of oxygen given infants of single birth and the incidence of cicatricial retrolental fibroplasia.

The groups of infants were so selected that the average stay in oxygen for each group was approximately the same. It may be seen that the incidence of retrolental fibroplasia was approximately the same in groups of infants of single birth who were kept in an oxygen-enriched environment for similar periods of time whether the average concentration was 50 or 35 percent.

The second slide represents infants of multiple birth, primarily all twins. Again, in the infants who received short stays in oxygen, namely, 1.6 days and two days, the incidence of retrolental fibroplasia was not dependent upon the average concentration of oxygen employed in the range of 33 to 50 percent. There is an increased incidence in those infants who had 12 days in oxygen, as shown by the upper line in this slide. The number of infants, however, is small.

With respect to those infants who received oxygen in concentrations under 40 percent after the first 48 hours of life, we found an over-all incidence of about six percent. There were six cases out of 104 that developed cicatricial retrolental fibroplasia. Of the total number of infants who developed cicatricial retrolental fibroplasia in the whole study, namely, 47 out of 596 live premature infants, six of the 47 never received oxygen above 40 percent. I believe it was in part because of these observations that Dr. Patz undertook his more quantitative study in animals.

I can only emphasize that the use of oxygen even at concentrations below 40 percent is accompanied by appreciable risk of developing cicatricial retrolental fibroplasia, the severity of which is indistinguishable in human beings from that in infants who received concentrations above 40 percent.

One final word in regard to the withdrawal problem: again, as Dr. Patz found, in human beings we have very good evidence to show that if one prolongs the stay in oxygen in order to withdraw the infant from the oxygen slowly, the incidence of the disease increases.

Dr. George K. Smelser: This study, which has been carried on by Dr. Patz and a number of other investigators over the world, was provoked by the occurrence of a serious clinical entity. The answer has finally come out of an investigation of a very fundamental aspect of biology. I hope that we do not forget this.

* Kinsey, V. E., et al.: Retrolental fibroplasia. Arch. Ophth., **56**:481-543, 1956.

The behavior of endothelial cells, of blood vessels in general, in a milieu of a raised oxygen tension, I believe is of importance not only to the ophthalmologist but to biologists generally. Has the author or perhaps others in this group investigated other loci than the eye, or have they been a bit narrow minded and looked only in the vitreous? How do the vessels behave in other parts of the body of these animals?

Other organs were also subjected to this high oxygen tension. If my memory serves me correctly, cases of retrolental fibroplasia have lesions in regions other than the eye. Do the authors ever obtain their counterpart in other organs of their experimental animals? If there are no observations of pathology in other places in the body, isn't it odd that vitreous and retinal vessels respond in such a spectacular manner, whereas those in other places in the body do not?

DR. NORMAN ASHTON (London): It is quite impossible to translate these animal findings into terms of human findings, and the first thing we must accept is what Dr. Kinsey tells us about the behavior of the human infant. That does not correlate very well with what is to be found in all animal experiments.

In the case of the kitten, and presumably also in other animals, I think the proliferative phase is dependent upon the irreversible vaso-obliteration which is produced by oxygen. In direct observations in the kitten, by putting in a window and administering increasing concentrations of oxygen one can find out exactly the percentage which produces vaso-obliteration.

In the kitten it is undoubtedly about 40 percent, though in our early experiments we had found in the kitten that a small proportion of these animals developed lesions in 35-percent oxygen. Certainly below that level we have never produced obliterative changes in the vessels which, in my opinion, are essential to the production of the subsequent lesions; that is, if there is no irreversible vaso-obliteration then there will be no proliferation. I think this means that the proliferations are not the result of oxygen per se.

I would like to ask both Dr. Patz and Dr. Kinsey how sure we can be of these concentrations in the eyes. How confident can one be that these percentages are the constant reading in all these experiments and in the clinical investigations?

DR. DAVID JOHNSON (Detroit): In the last year and a half I have had the opportunity to see five persons with retinal detachment who were premature babies of 2.5 to 3.0 pounds. One eye in three of these patients was lost, having had to be enucleated. The other eye presented retinal detachment. These people had had no oxygen and, at the equator in the temporal portion of the eye, there was a glial tissue proliferation with obliteration of the vessels, with the production of hole formation and retinal detachment. This is undoubtedly a minor form of retrolental fibroplasia.

DR. LANGHAM (London, England): Physiologically, the finding that 40-percent oxygen can induce changes in the retinal capillaries is very interesting, but rather mysterious. In an animal breathing air (20-percent oxygen) the maximal tension in the arterial blood will be 150 mm. Hg and the oxygen capacity of the blood approximately 20 vol. percent. When the oxygen content is increased to 40 percent the maximal oxygen tension in the arterial blood will be 300 mm. Hg but the oxygen capacity of the blood will be increased by only 0.5 vol. percent. Thus the amount of oxygen carried to the eye is increased very slightly in these conditions. We are left with the explanation that it is the rise in oxygen tension from 150 to 300 mm. Hg that causes the retinal changes. There is, however, the added difficulty that oxygen is lost from the arterial blood before reaching the capillaries of the eye and as it would only need a loss of one vol. percent of oxygen to bring the tension right down into the normal range it becomes questionable whether the oxygen tension in the capillaries of an animal breathing 40-percent oxygen would be significantly above normal. I would, therefore, like to ask Dr. Patz whether he feels the retinal changes are due to the effect of increased oxygen tension per se within the eye.

I should also like to ask whether the experiment has ever been made in which the oxygen tension was doubled by increasing the atmospheric pressure rather than adding extraneous oxygen, and if so were the same retinal changes found?

DR. DAVID COGAN (Boston): I would like to bring up something that is only indirectly related to Dr. Patz' thesis, and that is in regard to these changes in the retina—the proliferation of cells about the vessel walls.

With the interest in retrolental fibroplasia a few years ago, there was a rash of papers showing quite properly (by Dr. Heath and Dr. Ashton and others) the characteristic cellular proliferation of the vessels in the periphery of the retina.

Although certainly they are found with retrolental fibroplasia, the implication is that they are pathognomonic of retrolental fibroplasma, and I, for one, have been disappointed in the number of other conditions (congenital and miscellaneous) which also show a proliferation of this vessel wall in the periphery of the retina.

The most marked case I have ever seen was in a youngster with glioma of the optic nerve. I would like to know how characteristic these changes are of retrolental fibroplasia.

Perhaps Dr. Patz doesn't care to answer this, but it seems to me it is related to the general demonstration, and I would be interested in hearing also the comments of Dr. Heath in that regard.

DR. SZEWCZYK (St. Louis): I think there is one point which was not made quite clear. First, these changes which Dr. Patz described occurred after the animals were taken away from oxygen, and not while they were in oxygen. This means that these are changes of hypoxia and not of oxygen toxicity.

We know that there are babies who have de-

veloped retrolental fibroplasia in whom oxygen has never been used. I have seen some of these babies myself. It would be impossible to explain this disease on an oxygen-toxic basis if these babies developed it without any use of oxygen, but it is possible to explain it if we remember that this disease is caused by a sudden diminution of available oxygen to the baby after it has been acclimatized to an enriched atmosphere. It is quite possible that after birth, or during the time of birth or perhaps even before birth, the baby's oxygen supply may be so reduced that changes of retrolental fibroplasia may take place.

I also have to take issue with Dr. Patz' statement of gradual withdrawal. If you will remember, about five years ago I read a paper before a similar group on retrolental fibroplasia. At that time I stated that it was due to the rapid withdrawal of babies from oxygen after they had been acclimatized to an enriched atmosphere. I also found that it required five days of exposure to oxygen, so the baby would suffer if suddenly removed from it. If he were exposed from one to three days, sudden removal did not seem to make these changes appear.

For the last six years we have had the opportunity to see hundreds of premature babies, and our policy has been that if they have been kept in oxygen for any reason at all for over five to six days, they must be withdrawn very slowly.

If this disease is caused by rapid withdrawal or sudden withdrawal, then we have no answer for this problem but very gradual withdrawal. We know that it takes a normal adult approximately seven to 10 days to become acclimatized to a rarefied atmosphere. By frequent observations we have found that premature babies probably react in the same way.

To us, gradual withdrawal means this: If a baby has been acclimatized to a 40-percent oxygen concentration, his concentration is reduced over a period of 14 to 21 days. If the concentration has been higher we reduce the concentration over a much longer period of time. We have had many babies who were given continuous oxygen for periods of three to four months. If the prolonged administration of oxygen were the only cause of retrolental fibroplasia, all of those babies (and, incidentally, all of them were from the very small weight group) should have developed retrolental fibroplasia.

On a 30- to 40-percent range of oxygen, rapid withdrawal also is dangerous. We have had some babies who developed retrolental fibroplasia who had never been given more than 30 percent oxygen concentration. It is also our policy that, if a baby has been kept in the range between 30 and 40-percent oxygen for over seven days, we reduce their concentration very slowly.

DR. ARNALL PATZ (closing): I believe I will pick the questions that I may have even a remote answer to, and possibly Dr. Kinsey can tackle the more difficult ones.

Dr. Smelser raised the question of whether we had looked into the possibility of other vessels or other organs being affected by oxygen. Our first group of animals was subjected to as extensive a study as we could obtain, and especially the brain vessels were studied. The injected brains of the animals that showed the typical lesions in the eyes were studied in the neuropathology section at the Army Institute of Pathology, and their appraisal of the material submitted was that there was no difference in the control and oxygen-treated animals.

I don't think it is an odd situation that the retinal vessels are specifically involved and the vessels elsewhere are not, because I think there is no other place in the body, except possibly one zone in the kidney, that at four-months gestation does not have a well-defined blood supply. In the retina there are no vessels whatsoever until the fourth month.

Another thing that I believe is important as to why the eye is specifically involved—at least the retinal vessels—is the peculiar architecture of the eye, namely, that a rich bed of vessels in the choroid lies in direct apposition to the retina. Dr. Ashton has shown very nicely with his window technique that, if you detach the retina from its attachment to the choroid, the vasoconstrictor effect of oxygen is no longer noted; in the detached retina the vessels are of normal caliber or dilated, whereas the retina that is against the choroid in normal position is constricted with oxygen.

In answer to Dr. Ashton's question of being sure of the concentration in the incubator, I don't think we can be too critical of the concentration used if we are willing to accept a range of four to five-percent as an adequate control. The Isolette has a sleeve which, when any other gadget is to be introduced into the chamber, the total oxygen concentration in the chamber is not appreciably disturbed. So, I think the levels that are reached maintain a reasonably stable concentration.

Our determinations were made with the Beckmann analyzer, which is accurate within ± two-percent concentration and, when we were working especially with the lower concentrations, we found that, by using precision flowmeters, we rarely had a range greater than two or three percent above or below what we were trying to obtain. When we got to concentrations of 60 or 80 percent, there was more fluctuation, and there might be a range of as much as six or eight percent above or below the levels that were desired.

Dr. Johnson raised one question about seeing detachment equatorially in babies who had received no oxygen. I do not know how Dr. Ashton feels, but I certainly am not convinced that oxygen is the only factor that could produce these proliferative changes in the premature infant. Certainly one could very quickly postulate many hormonal factors that are different in an infant who is born prematurely and who is raised in extrauterine environment from those in the normal infant who goes to term in utero.

If you want to stretch the oxygen theory, I think you are within the hypothesis that oxygen as the

key factor explains these cases, because in utero the arterial oxygen concentration of the premature infant is only about 50 percent. If a premature infant is born and raised in room air with no added oxygen, there is a rise in arterial oxygen within approximately one hour after birth to about 90 percent saturation. That is with no added oxygen. I think this is explained by the closure of the foramen ovale and the cessation of the admixture of the venous and arterial blood that goes to the head.

We just don't know the answer to these cases that occur when no added oxygen is used; possibly some other factors may ultimately be found to explain them.

To Dr. Langham's statements I have no answer. Our experiments do not give any clue as to why these very small increments of increase in actual tissue oxygen pressure can explain such extensive changes in the normal vascularization of the retina as are seen in the oxygen-abnormal eye. Possibly Dr. Kinsey or Dr. Ashton may care to comment on that.

In experiments on oxygen poisoning, the pressure in atmospheric oxygen is increased; simply by raising room air to three atmospheres you get an equivalent of 60-percent oxygen. The so-called enzyme changes that are produced are reported to occur with increasing room air to equivalent high oxygen concentrations.

Dr. Cogan questioned the assumption that these proliferative changes might be pathognomonic of retrolental fibroplasia. I think that is a valid criticism of accepting these animal lesions as necessarily pure retrolental fibroplasia. At least it simulates the lesion that has been described as pathognomonic of the early changes, and I think the later changes. The eruption of vessels into the vitreous is not appreciably different from that occurring in diabetics and the way the vessels erupt, except immature vessels, seems to be the determining factor in the oxygen-induced lesions.

Dr. Szewczyk has raised the question of the removal of oxygen and gradual withdrawal. I have no clinical data on gradual withdrawal from oxygen. In our clinical study, before we had any knowledge that the experimental lesion could be produced, babies were kept in high oxygen continuously for 30 days at levels of 60 to 70 percent.

The thing that is striking here is that we saw several cases of early active disease develop while the babies were still in oxygen. At this time we were not aware of the acclimatization factor that Dr. Szewczyk mentioned. I do think that acclimatization does occur, and certainly the withdrawal of oxygen from the animal is a very important factor. It determines, at least in our hands, the time of appearance of the lesions. We have not felt, however, that a gradual withdrawal had any ultimate effect on the appearance of the lesions, based on the studies in mice.

I think there are other investigators who have seen retrolental fibroplasia occur while babies were getting continuous oxygen. I know Dr. Day, who was formerly in Dr. Reese's nursery, had 15 cases that were recorded while the infants were still getting oxygen. I think cases occurred in Dr. Kinsey's co-operative study.

So, from the clinical data we can state very definitely that it is not necessary to have abrupt withdrawal or so-called acclimatization and relative hypoxia occur for the disease to be produced. I would be willing to agree that rapid withdrawal or this relative hypoxia can influence and may accentuate the basic oxygen lesion.

DUAL RESPONSE OF THE LIMULUS LATERAL EYE TO ELECTRIC STIMULATION*

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I. INTRODUCTION

Hartline, Coulter, and Wagner[1] reported in 1952 that the lateral eye of Limulus can respond to electric stimulation, as well as to light. They found that passage of an electric current through the whole eye could produce a discharge of impulses in the optic nerve fibers. Furthermore, depending on the direction of the current, the response of the eye to illumination was found to be inhibited or facilitated by the current. Later, Sten-Knudsen showed that a previous light stimulus increased temporarily the electric excitability of the eye.[2]

The present study is a continuation of the above cited work, and it was undertaken to elucidate with an improved technique further details of the electrical response mechanism.

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II. Method

The method was very similar to that used in previous studies.[1,2] A lateral eye with three to five centimeters of the optic nerve intact was excised from an adult Limulus, and mounted in a special chamber as diagramed in Figure 1. The optic nerve (o) was led over a barrier (b) into a saline pool (III). One (d) of a pair of Ag-AgCl wick electrodes was placed in the pool. A small bundle of nerve fibers (f) was dissected loose at the cut end of the optic nerve and raised from the pool onto the second of the electrodes (e). With this arrangement action potentials occurring in the small bundle (f) produced between the electrodes (d) and (e) voltage differences which could be amplified and recorded.

A time constant of 0.001 sec. was used in the amplifier to avoid displacement of the baseline during passage of current through the eye. The number of fibers in the bundle was reduced until only a single nerve fiber remained active in the bundle, judging from the regularity of discharge and uniformity of the size and shape of the action potentials.

Electrically isolated pools (I) and (II) were applied respectively to the cornea (c) and the back of the eye (a). Large Ag-AgCl electrodes (g) and (h) were connected through seawater-agar bridges (i) and (j) to each of these pools, and a current source connected across these electrodes. A stimulating current of controlled strength and duration could then be passed through the eye in either direction from one pool to the other.

A small beam of light (k) was focused on the corneal facet of the ommatidium

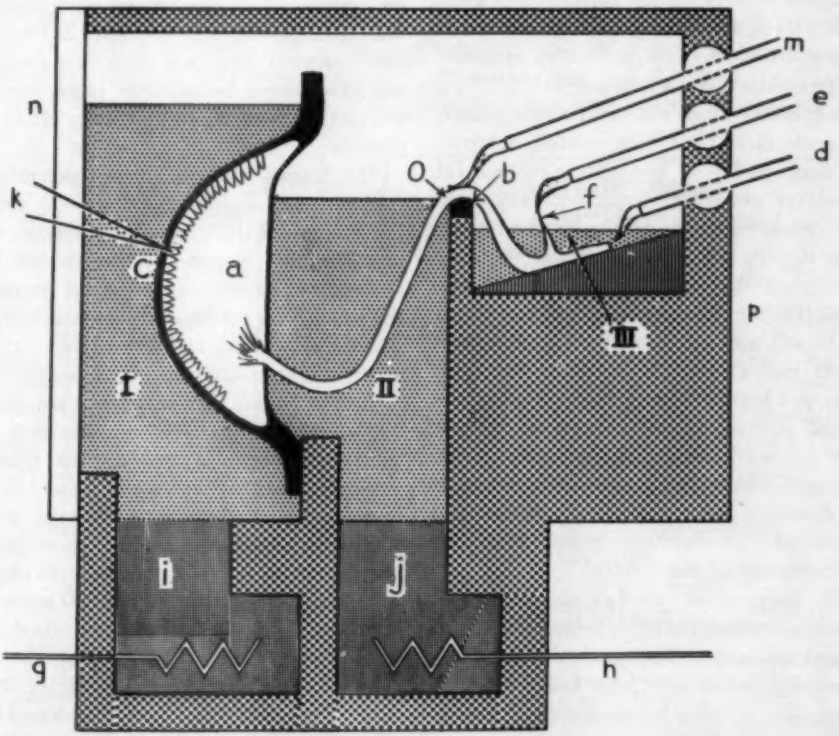

Fig. 1 (Lipetz). Experimental arrangement for measurement of nerve impulses from the Limulus lateral eye. Description in text.

which gave rise to activity in the particular nerve fiber under observation. This permitted an exclusive stimulation by light of that ommatidium and fiber.

Special precautions were needed to keep the artefact voltage, produced at the pick-up electrodes (d and e) by the stimulating current, from being so large as to mask the action potentials. First, a grounded, wick-type Ag-AgCl electrode (m) was placed on the optic nerve (o) where it crossed the barrier (b) between pools (II) and (III). This also served to keep moist the portion of optic nerve not immersed in a pool. Second, the electrical resistance between the ground electrode (m), the pick-up electrodes (d and e), the current source, and each of the pools (I, II, III) had to be kept greater than 100 megohms. For this reason the entire chamber was made of lucite except for the front glass window (n). Paraffin was used to coat the lucite and seal all joints, as well as to mount the eye and the glass window.

Pool I was filled with artificial seawater, and pools II and III with aerated, defibrinated Limulus blood. The bottom of pool III sloped up toward the back and was lined with paraffin-coated black bakelite. This made it easier to see and dissect the fiber bundles of the optic nerve. Since it was found that the optic nerve became inactive if the animal was dehydrated, all Limuli were placed into (artificial) seawater for three hours just before the experiment. Ommatidia located at the extreme margin of the eye were not used because the stimulating current path through such ommatidia would be asymmetrical about the axis of symmetry of the ommatidium and so would complicate interpretation of the results.

The preparation chamber was fitted with a moisture-proof cover to prevent drying of the eye and nerve. The usual life of a single fiber preparation was four hours at 22°C. and longer at lower temperatures.

Electrical polarization was minimized by alternating the current direction every 10 seconds.

The measurement errors were: for latent periods, ± 2.0 milliseconds; for stimulus duration, ± 0.5 percent, and for relative current strengths, ± 0.25 percent.

III. Results

When a constant current was applied with the cathode at the cornea, action potentials were recorded from the optic nerve fiber as illustrated in record A of Figure 2. This response is typical of those observed by Hartline, Coulter, and Wagner.[1] As the current strength was increased, the latencies of all action potentials gradually decreased. However, a new finding was that, when a critical current strength was reached, the latent period of the first action potential in the response suddenly became very short, as illustrated in record B of Figure 2. Previous experimenters have not been able to make this observation because the large stimulus artefact masked any short latency action potentials.

The latency of each action potential in the response is plotted in Figure 3 against the strength of the stimulating current which produced that response. It can be seen that at the critical current strength the latency of the first action potential became suddenly reduced and the strength-latency curves showed a discontinuity.

It was found that in every case the response could be classified into one or a combination of both of two distinct types of response: the "early response," consisting of action potentials having an observed latency of less than 40 msec; and the "late response," consisting of action potentials having an observed latency of more than 90 msec. The observed latency included the conduction time of the nerve impulse along the optic nerve fiber from the eye to the recording electrodes; between 15 to 40 msec. Therefore, the true latency of the "early response"

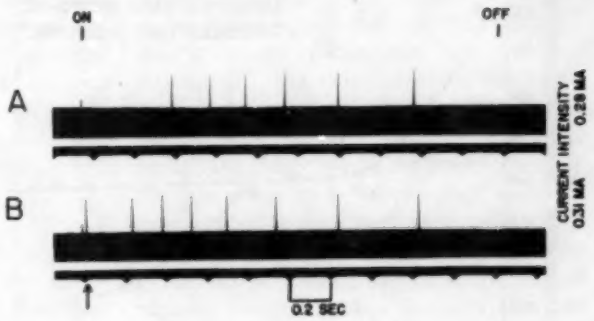

Fig. 2 (Lipetz). Records of action potentials from a single active optic nerve fiber of the Limulus lateral eye during passage of an electric current through the whole eye. The beginning and end of the 2.0 second stimulation period are marked "on" and "off." The cathode was at the cornea. Time marks at 0.2 second intervals appear at the bottom of each record. Record A shows the response to 0.28 ma. current. Record B shows the response to 0.31 ma. current. Note the very short latency action potential ("early response") indicated by the arrow at the bottom left of the record.

was many times smaller than that of the "late response."

The strength and duration values of the stimulating current for threshold responses of a typical single fiber preparation are plotted in Figure 4. Along line AB lie the values of current strength and duration which just produce a "late response" consisting of a single action potential. Upon increasing the current strength a second threshold (BD) is reached at which there appears in addition an "early response" consisting of a single action potential. These responses are illustrated by the records of A in Figure 5. Along line BE lie the threshold values of current strength and duration for short electrical stimuli which produce an "early response" consisting of a single action

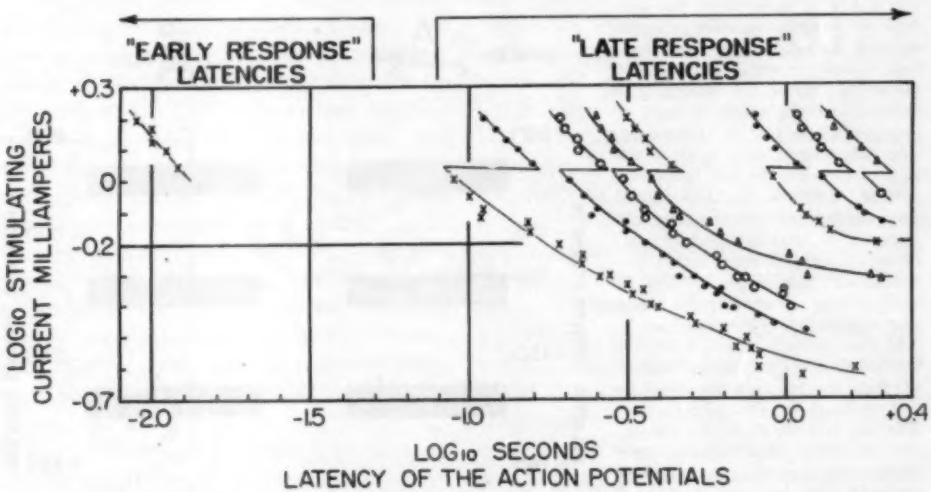

Fig. 3 (Lipetz). Latencies of the first eight action potentials of the discharge from a single active optic nerve fiber at various stimulating current strengths. The current was passed for 2.0 seconds through the whole Limulus eye, with the cathode at the cornea. X indicates the first and fifth action potentials; ●, the second and sixth; O, the third and seventh; Δ, the fourth and eighth. Note the very large change in latency of the first action potential, and the smaller changes of the other action potentials at the critical current strength of 1.1 ma.

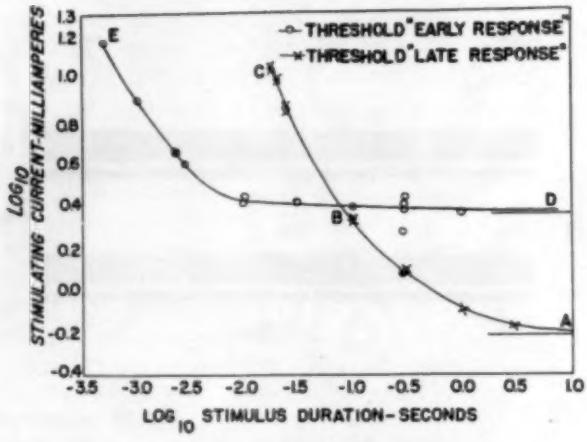

Fig. 4 (Lipetz). Threshold strength-duration curves for the two types of response to passage of electric current through the whole Limulus eye. The cornea was kept cathodal. Curve ABC represents the threshold for the "late response," and has a rheobase (indicated by a short horizontal line at the right) of 0.6 ma., and a chronaxie (indicated by a filled circle) of 300 msec. Curve EBD represents the threshold for the "early response," and has a rheobase of 2.8 ma. and a chronaxie of 2.4 msec.

potential. Upon increasing the current strength a secondary threshold (BC) is reached at which there appears in addition a "late response" consisting of a single action potential. These responses for short current durations are illustrated by records B in Figure 5.

All responses described above were for

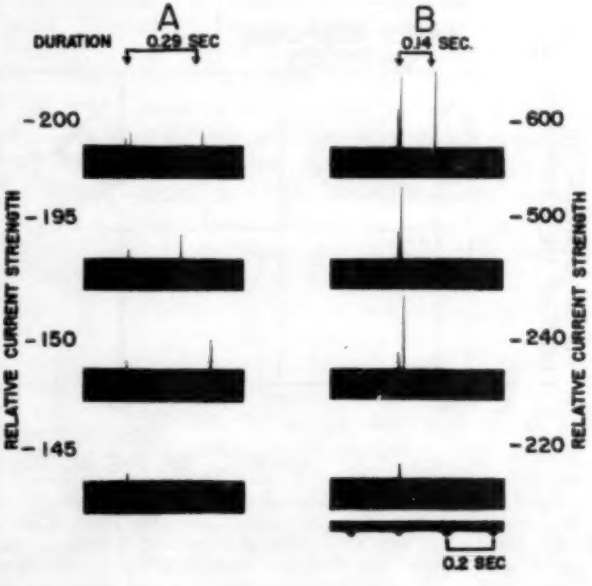

Fig. 5 (Lipetz). Records of action potentials from a single active optic nerve fiber of the Limulus lateral eye in response to the passage of cornea-negative current through the whole eye. The duration of the current is indicated by the arrows above the records. The relative current strength used is indicated at the side of each record. The two bottom records show the lack of response to just subliminal current. The two records second from the bottom show the threshold response to slightly stronger currents: a long latency action potential, or "late response" for A, and a very short latency action potential, or "early response" for B. The two records third from the bottom show the decrease in latency of these responses when the current strength is increased further. The top records show the appearance of both types of response when the current strength is made slightly stronger. The records of A were made from a different optic nerve fiber than those of B, but the two sets were typical of the responses of any one of the single fiber preparations. Time marks of 0.2 second are shown at the bottom of the B records. Artefacts were removed from the records by retouching.

electrical stimulation with the cornea made cathodal. When the cornea was made anodal, only the "early response" could be obtained during current flow. The threshold strength-duration curve for the "early response" with cornea anodal differed from that with cornea cathodal only in the slightly higher current threshold shown by most preparations.

IV. Discussion

The existence of two separate threshold strength-duration curves for the two different types of response ("early" and "late") to electrical stimulation of the whole Limulus lateral eye indicates that two separate mechanisms are involved. The "early response" mechanism shows a higher rheobase and a very much shorter chronaxie than does the "late response." The fact that the two strength-duration curves show no discontinuity where they intersect (point B in Figure 4) indicates that the two mechanisms act essentially independently to produce the recorded action potentials in the nerve fiber.

The abrupt changes in response as the stimulating current strength was changed can now be explained as a change in which response mechanisms were being activated by the current. Thus, if at the current duration used, the current strength was above the threshold represented by line CBA but below that represented by line EBD, only the "late response" mechanism was activated. If the current strength was above the threshold represented by line EBD but below that represented by line CBA, only the "early response" mechanism was activated. If the current strength was above the two thresholds, then both response mechanisms were activated.

If confusion is to be avoided, all work on electric stimulation of the Limulus lateral eye should specify which of these response mechanisms was activated by each stimulus. The previous work of Sten-Knudsen[2] was done under conditions which produced only "early responses." However, the work of Hartline et al.[1] was done with a wider variety of conditions and it is uncertain which response mechanisms were involved.

Although, as stated above, the "early response" mechanism and the "late response" mechanism act essentially independently, there is some evidence of their interaction in the generation of nerve impulses in the optic nerve fiber. In Figure 3, the lines connecting the data points were drawn so that the wider time interval between the fourth and the fifth "late response" action potentials was maintained above, as well as below, the critical current intensity. This assumes that the relative spacing of the "late response" action potentials was determined by the "late response" mechanism and was not affected by activity of the "early response" mechanism. On this assumption, the curves of Figure 3 can be interpreted to mean that when the "early response" mechanism became active, an additional action potential, of very short latency, was produced; and the latencies of all the "late response" action potentials were slightly increased. This effect of the "early response" mechanism on the absolute latencies of the "late response" action potentials is evidence of interaction of the two mechanisms.

The existence of two response mechanisms implies that there are two different sites and structures in the Limulus lateral eye which are especially sensitive to the passage of electric current through the eye. That these two response mechanisms can interact with the normal light-response mechanism of the eye has been shown by previous workers.[1,2] Therefore, identification of the sites and mechanisms of these two electric responses would also give information on some of the structures and processes involved in the normal light response. Such an identification will be attempted in a later paper[3] on the basis of the above results and additional experiments.

V. Summary

An improved preparation of the Limulus lateral eye is described by means of which a

stimulating electric current may be passed through the whole eye. The response consists of action potentials recorded from a single active fiber of the optic nerve.

The responses to whole-eye electric stimulation were found to be produced by two distinct mechanisms, the "early" and the "late." These response mechanisms differed in that:

a. The action potentials of the "early response" had a much shorter latency than those of the "late response."

b. The threshold strength-duration curve for the "early response" had a higher rheobase and a much shorter chronaxie than did the curve for the "late response."

c. Both mechanisms could produce responses during current flow when the cornea was cathodal, but only the "early response" mechanism could do so when the cornea was anodal.

Although these response mechanisms were essentially independent, the "early" affected the latencies of nerve impulses produced by the "late."

Department of Ophthalmology (10).

ACKNOWLEDGMENTS

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DISCUSSION

DR. WERNER K. NOELL (Buffalo): The interest of the basic sciences in vision has recently been focussed more and more upon intracellular events and structural substrates which generate excitation. Studies on the invertebrate eye have greatly contributed to the foundation upon which retinal electrophysiology is based. We can rightfully expect that the invertebrate eye will finally prove to be a decisive test for the basic problem of vision, as we see it today.

I wish I were able to draw a structural analogy between the invertebrate and the vertebrate photoreceptors because this would probably best illustrate the fascinating scope of the problem and the experimental advantage of the invertebrate eye. My difficulties are with the invertebrate eye in general and that of the horseshoe crab in particular.

As I understand it, one ommatidium—which we may compare with one rod cell or group of them—consists of eight to 20 cells. From each of these cells emerges a fine fiber which seems to connect with the next neuronal layer. Each of these cells—the retinular cell—carries the equivalent of an outer limb, the so-called rhabdomere.

Electronmicroscopic studies by Fernandez-Morán and by Wolken have shown that the fine structures of outer limb and rhabdomere are essentially identical. The curious fact, however, is that the fibers which emerge from the retinular cells are electrically inactive, at least they do not have spike activity. The activity which one records from the fiber bundle of an ommatidium in response to illumination is that of only one fiber and this fiber is the axon of the so-called eccentric cell. The soma of the eccentric cell lies between retinular cells but it has a special distal process which runs in the axis of the ommatidium.

In familiar terms, one may visualize the ommatidium as an orange, the retinular cells are the segments of the orange and the axial region of the orange contains the outer limb equivalents and the peripheral process of the eccentric cell.

There have been discussions whether the eccentric cell is a ganglion cell or a receptor cell. I could not find evidence that it has a photoreceptor surface but I would like to compare its peripheral process with the inner limb of the vertebrate receptors where the primary events of vision are probably geared into an electrical process.

I wonder whether Dr. Lipetz could carry on from here and tell us upon which structure of the Limulus eye the electrical current might impose

the phenomena he analyzed. I, furthermore, would be curious to know the suggestions of an expert on the functional conversion between retinular cell and eccentric cell because it is at this point where a superficial analogy between vertebrate and Limulus receptors falls down.

To be specific: Are retinular cells and eccentric cells merely a variation of a receptor cell or are they the equivalents of a modified vertebrate pigment epithelial cell and receptor cell?

DR. LEO E. LIPETZ (closing): I think the best thing to do would be to show a slide that I did *not* bring along for this lecture.

This shows something of the anatomy of the ommatidium. Here we have the cornea. This is the lens of the cornea. This whole structure that I am outlining is the ommatidium. Here are the cells grouped as the segments of an orange, and right through the center extends the distal process of the eccentric cell. Here is the cell body, and here is its axon.

Dr. Noell's discussion and his suggestion that retinular cells correspond to pigment epithelial cells is a very intriguing one, because we do know that the retinular cells are pigmented and they are epithelial in origin. We know that in the vertebrate eye there is a layer of pigmented epithelial cells which serves as one source of electric voltages used in the process of reception, and we know this is true also in the cochlea of the vertebrate. It looks as if that may be true in the invertebrate eye as well.

It may be that these retinular cells do correspond to the pigment epithelial cells. If such is the case, then we would expect to find a constant voltage, a resting potential, between the inside and outside of the layer of cells. This is something that has not been tested, but the question has now been raised and it can be tested.

The function of the distal process is again open to question, but certainly it is a very specialized synaptic entity. There it is, next to the rhabdomere, the rhabdomere surrounding it. As Dr. Noell pointed out, the rhabdomere here corresponds to the outer limbs of the vertebrate retina. The distal process may simply be specialized to accept the electric current from these outer structures so that the current can flow through and then perhaps out in the axon hillock region, back again to the retinular cells, and so stimulate a sensitive portion of the membrane at the axon hillock. That may be the function of the distal processes. However, it may be that they have another function corresponding even more closely to that of the inner limbs, and this I simply cannot discuss.

To say more about the Limulus eye right now would be pure speculation. I think I will leave that for another time.

ON THE SOLUTION OF THE DIFFERENTIAL EQUATIONS PERTAINING TO AQUEOUS HUMOR DYNAMICS*

S. I. ASKOVITZ, M.D.
Philadelphia, Pennsylvania

A number of papers have been published in the ophthalmologic literature in which the methods of differential equations were applied to the study of aqueous humor flow.[1–5] The original work of Kinsey and collaborators was of fundamental importance in this field. The mechanisms of secretion, diffusion and ultrafiltration, and so forth, were subjected to mathematical analysis, and experimental data on a variety of test substances were reported. It was shown that a comparison of steady-state levels alone does not suffice to identify the mechanism of transfer of a substance from the blood plasma into the aqueous humor.

Some years later, after a technique of withdrawing samples of aqueous humor directly from the posterior chamber had been perfected by Kinsey, it became possible to analyze the results on ion transfer in greater detail. It was concluded that a secretion hypothesis best fitted the data for sodium, while a diffusion explanation was preferable for thiocyanate.[4]

A further contribution toward our understanding of the ocular circulatory dynamics was made by Friedenwald and Becker.[5] The "reservoir" compartment, representing a combination of crystalline lens, vitreous humor, and so forth, was given a more active role in the equations.

* From the Department of Research, Wills Eye Hospital and the Ophthalmology Research Laboratory, Albert Einstein Medical Center, Northern Division. This work was supported in part by a research grant from the Weinstock Fund.

Formulas containing several exponential terms were used, and previously available experimental data were fitted graphically by the compound exponential type of equation. This made it possible to include all of the recorded values in the analysis, whereas some portions of the observed data had previously not corresponded well with the results of simpler curve fitting. However, the assumption was still made that the concentration of the test substance in the circulating plasma remained constant, in order to simplify the theoretic details.

Mathematical analyses quite similar to those applicable to the ocular circulation have occupied the attention of scientists in other fields for many years. Physiologists studying the cardiac output and general peripheral circulation[6–8] have used a variety of radioactive and other tracer substances. Papers discussing the abstract mathematical and physical aspects of the resulting differential equations have also been published.[9–11]

It is the purpose of the present note to extend the previous work along the following lines. First, the differential equations can be solved under more general assumptions than have previously been made. For example, equations will be set up for the case in which a substance injected into the circulating plasma disappears gradually, according to an exponential decay law, or where the level approaches a limiting value asymptotically. This would seem to represent the facts better than to assume the maintenance of a fixed concentration.

Secondly, it is possible without great difficulty to enlarge the compartment system and to consider exchanges between more than three compartments. Four or more of circulating plasma, anterior chamber, cornea, posterior chamber, lens, and posterior reservoir may be included.

Third, the graphic methods hitherto used in fitting single or multiple exponential curves are subject to the errors inherent in the customary fitting of straight lines on semilogarithmic charts. Instead, an exact procedure can be substituted for the arbitrary one now commonly used. By application of the principles of least squares, it is possible to obtain a unique solution for the equation of best fit, not subject to any individual's decision. The cumbersome calculations can generally be replaced by a graphic device recently described.[12]

Before entering into the general discussion of multicompartment systems, it would be well to review briefly the simple two-chamber model from a mathematical point of view. Some of the experimental data on aqueous humor can be analyzed by means of these more basic concepts.

Case 1

Let us assume that a substance is being transferred from Compartment Q to Compartment R. This may be thought of as taking place across a biologic membrane M, or more directly, through some opening from one vessel to another (Fig. 1). Perhaps the simplest type of mathematical relationship would be the case in which the rate of transfer of the material, or the rate of increase of concentration of the substance in Compartment R, is directly proportional to the concentration of the substance in Compartment Q. For the present, it is assumed that none of the substance leaves Compartment R. If the concentration of the substance is held at a constant level in Compartment Q (either by continuous slow replacement, or by reason of the large size of Q in comparison with the relatively small

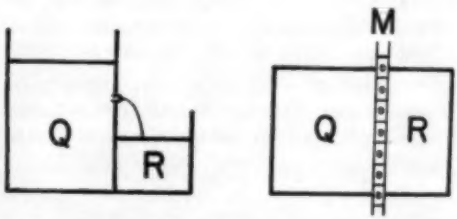

Fig. 1 (Askovitz). General two-compartment system, illustrating transfer from Chamber Q to Chamber R by flow (or secretion) on the left, and by diffusion across Membrane M on the right.

outflow), then we may proceed to set up and solve the equations in a very direct manner. Capital letters will be used to represent the concentration of the test substance in the respective compartments. The subscript 0 will refer to the initial concentration at time $t=0$, and the subscript ∞ to the ultimate limiting value (where this is applicable).

$$\frac{dR}{dt}=cQ=cQ_0. \tag{1}$$

Integrating, $$R_t=cQ_0t+k. \tag{2}$$

Replacing t by zero (that is, substituting the initial values),

$$R_0=k.$$

Therefore, $$R_t=cQ_0t+R_0. \tag{3}$$

CASE 2

Now let us assume that the concentration of the substance in Compartment Q is falling according to an exponential law:

$$Q=Q_0e^{-at}.$$

This leads to a solution containing the same type of exponential term.

$$\frac{dR}{dt}=cQ=cQ_0e^{-at}. \tag{4}$$

Integrating, $$R_t=-\frac{c}{a}Q_0e^{-at}+k. \tag{5}$$

Replacing t by zero,

$$R_0=-\frac{c}{a}Q_0+k,$$

$$k=R_0+\frac{c}{a}Q_0.$$

Substituting this value of k into equation (5), we have

$$R_t=R_0+\frac{c}{a}Q_0(1-e^{-at}). \tag{6}$$

Alternatively, the constant of integration k may be evaluated by proceeding in equation (5) to $t=\infty$ (that is, substituting the ultimate steady-state values):

$$R_\infty=k.$$

Substituting back into equation (5),

$$R_t=R_\infty-\frac{c}{a}Q_0e^{-at}. \tag{7}$$

It is possible to eliminate the initial value Q_0 from the solution altogether by substituting $t=0$ in equation (7) or $t=\infty$ in equation (6). In either case, we obtain

$$\frac{c}{a}Q_0=R_\infty-R_0$$

and therefore

$$R_t=R_\infty-(R_\infty-R_0)e^{-at} \tag{8}$$

or

$$R_\infty-R_t=(R_\infty-R_0)e^{-at}. \tag{8'}$$

CASE 3

To assume that the concentration Q exponentially approaches a nonzero limiting value Q_∞ does not present any new difficulty. We may write

$$Q=(Q_0-Q_\infty)e^{-at}+Q_\infty,$$

with the resulting equation

$$\frac{dR}{dt}=c(Q_0-Q_\infty)e^{-at}+cQ_\infty. \tag{9}$$

By direct integration,

$$R_t=-\frac{c}{a}(Q_0-Q_\infty)e^{-at}+cQ_\infty t+k. \tag{10}$$

The constant of integration may be replaced in terms of the initial value R_0, obtaining

$$R_t=\frac{c}{a}(Q_0-Q_\infty)(1-e^{-at})+cQ_\infty t+R_0. \tag{11}$$

There could be no limiting value R_∞, since R becomes unbounded as t increases.

It may be noted that this solution includes the previous results as special cases. If the limiting value Q_∞ equals zero, then equation (11) reduces to equation (6). If, on the other hand, the exponent a is taken to equal zero, Q would remain constant, and equation (11) would reduce to equation (3) since $Q_\infty=Q_0$.

CASE 4

Thus far, it has been taken that there is a complete blockage to any outflow from Compartment R. If we now assume that there is again a constant source Q, but in addition a steady outflow from chamber R, represented by $dR/dt=-fR$, the resulting equation will contain an exponential term.

The rate of fall represented by the constant term in the exponent will be the same as the coefficient of outflow from chamber R.

$$\frac{dR}{dt} = cQ_0 - fR. \tag{12}$$

Solving by the standard methods of differential equations,

$$R_t = \left(R_0 - \frac{c}{f}Q_0\right)e^{-ft} + \frac{c}{f}Q_0, \quad \text{or} \tag{13}$$

$$R_t = R_0 + \frac{c}{f}Q_0(1 - e^{-ft}). \tag{13'}$$

Substituting $t = 0_\infty$, the exponential terms vanish, and the steady-state ratio is obtained:

$$\frac{R_\infty}{Q_\infty} = \frac{R_\infty}{Q_0} = \frac{c}{f}, \tag{14}$$

where c and f are the original outflow coefficients.

CASE 5

If, however, the concentration of test material in Compartment Q is assumed to decrease exponentially toward zero, we are led to a result which includes two different exponential terms. The differential equation is

$$\frac{dR}{dt} = cQ_0e^{-at} - fR, \tag{15}$$

and the solution would be of the form

$$R_t = ke^{-ft} + me^{-at}, \tag{16}$$

where m is a multiplier to be determined and k is an arbitrary constant depending upon the initial or boundary conditions. This becomes

$$R_t = \left(R_0 - \frac{cQ_0}{f-a}\right)e^{-ft} + \frac{cQ_0}{f-a}e^{-at}, \quad \text{or} \tag{17}$$

$$R_t = R_0e^{-ft} + \frac{cQ_0}{f-a}(e^{-at} - e^{-ft}). \tag{17'}$$

In general, it would be expected that a, the coefficient of decay of the test substance in Q, would be smaller (that is, representing a slower rate) than f, the coefficient of outflow from R, and therefore $f-a$ has been written rather than $a-f$.

CASE 6

Taking now the more general hypothesis of the concentration Q approaching a non-zero limiting value exponentially, with again an outflow from the second compartment proportional to the concentration R, we have

$$\frac{dR}{dt} = c(Q_0 - Q_\infty)e^{-at} + cQ_\infty - fR. \tag{18}$$

This leads to the integrated form

$$R_t = ke^{-ft} + me^{-at} + n, \tag{19}$$

where m and n are uniquely determined constants and k depends upon the initial values. The complete solution is:

$$R_t = \left[R_0 - \frac{c(Q_0 - Q_\infty)}{f-a} - \frac{cQ_\infty}{f}\right]e^{-ft} + \frac{c(Q_0 - Q_\infty)}{f-a}e^{-at} + \frac{cQ_\infty}{f}, \tag{20}$$

or

$$R_t = R_0e^{-ft} + \frac{c(Q_0 - Q_\infty)}{f-a}(e^{-at} - e^{-ft}) + \frac{cQ_\infty}{f}(1 - e^{-ft}). \tag{20'}$$

This result includes equation (13') if a is taken to be zero (then also $Q_\infty = Q_0$), and equation (17') if Q_∞ equals zero. It may be noted that even under the more general assumptions underlying the solution in equation (20'), we can still infer concerning the steady-state ratio that $R_\infty/Q_\infty = c/f$, as in equation (14) of Case 4.

Thus far, it has been assumed that the rate of transfer is not influenced by the amount of the substance in the second chamber. This corresponds mathematically to a hypothesis of either *flow* or *secretion*, and may suffice to explain a variety of observed data. However, for substances which do not follow this simpler type of transfer pattern, the next step in order of increasing complexity is to add a term representing the effect of the concentration in the second chamber. This is mathematically equivalent to an assumption of a *diffusion* mechanism.

Again, we may consider a number of

cases, assuming either a constant concentration in Q or an exponential drop toward zero, or an asymptomatic approach to a non-zero limiting value, and with or without continuous outflow from R. In most biologic instances, a proportional transfer out of chamber R would be applicable. However, it may be worth while to work through the examples with blocked outflow as a preliminary step.

CASE 7

Diffusion out of a chamber with concentration level held constant in the first compartment:

The equation

$$\frac{dR}{dt}=b(Q_0-R)=bQ_0-bR \tag{21}$$

has the same form as equation (12) of Case 4, if we replace c by b and f by b. The solution is therefore

$$R_t=R_0+Q_0(1-e^{-ft}), \tag{22}$$

with a steady-state ratio of unity ($c/f=b/b=1$).

CASE 8

Transfer by diffusion, with the amount of test material in the first chamber falling exponentially: Here the equation is

$$\frac{dR}{dt}=b(Q_t-R_t), \tag{23}$$

or

$$\frac{dR}{dt}=bQ_0e^{-at}-bR, \tag{23'}$$

which corresponds to equation (15) of Case 5. The solution may, therefore, be obtained by substituting b for c and f in equation (17'):

$$R_t=R_0e^{-bt}+\frac{bQ_0}{b-a}(e^{-at}-e^{-bt}). \tag{24}$$

CASE 9

Transfer by diffusion with concentration in first compartment falling exponentially, but to a nonzero limit Q_∞: The differential equation is

$$\frac{dR}{dt}=b(Q_t-R_t)$$
$$=b(Q_0-Q_\infty)e^{-at}+bQ_\infty-bR_t, \tag{25}$$

exactly analagous to equation (18), again with c and f both to be replaced by b. Hence the solution may be copied from equation (20'):

$$R_t=R_0e^{-bt}+\frac{a(Q_0-Q_\infty)}{b-a}(e^{-at}-e^{-bt})+Q_\infty(1-e^{-bt}). \tag{26}$$

The steady-state ratio, as under Case 7, will equal one.

It may be of interest to point out from the results of the last three cases, that as far as the mathematical aspects are concerned, the hypothesis of diffusion from a large reservoir compartment into a chamber with blocked outflow, leads to the same concentration curves as the assumption of proportional outflow from the second chamber, with entry from the reservoir compartment by flow or secretion.

Now, superimposing the more physiologic condition of continuous outflow upon the assumption of entry by diffusion, we are led to consider three further cases.

CASE 10

Transfer from first to second compartment by diffusion, and proportional outflow from second chamber; concentration in first chamber held constant (fig. 2A).

CASE 11

Transfer from first to second compartment by diffusion, and proportional outflow from second chamber; concentration in first chamber dropping exponentially to zero (fig. 2B).

CASE 12

Transfer from first to second compartment by diffusion, and proportional outflow from second chamber; concentration in first chamber decreasing exponentially toward a non-zero limiting value (fig. 2C).

More generally, let us assume that the material in question is being transferred from the first compartment to the second

Case 10.

$$\frac{dR}{dt} = b(Q_0 - R) - gR = bQ_0 - (b + g)R. \tag{A-1}$$

$$R_t = \left(R_0 - \frac{b}{b+g} Q_0\right)e^{-(b+g)t} + \frac{b}{b+g} Q_0. \tag{A-2}$$

$$\frac{R_\infty}{Q_\infty} = \frac{R_\infty}{Q_0} = \frac{b}{b+g}. \tag{A-3}$$

Case 11.

$$\frac{dR}{dt} = b(Q_t - R_t) - gR_t = bQ_0e^{-at} - (b + g)R. \tag{B-1}$$

$$R_t = R_0e^{-(b+g)t} + \frac{bQ_0}{b+g-a}\left(e^{-at} - e^{-(b+g)t}\right). \tag{B-2}$$

Case 12.

$$\frac{dR}{dt} = b(Q_t - R_t) - gR_t = b(Q_0 - Q_\infty)e^{-at} + bQ_\infty - (b + g)R. \tag{C-1}$$

$$R_t = \left[R_0 - \frac{b(Q_0 - Q_\infty)}{b+g-a} - \frac{bQ_\infty}{b+g}\right]e^{-(b+g)t} + \frac{b(Q_0 - Q_\infty)}{b+g-a}e^{-at} - \frac{bQ_\infty}{b+g}, \tag{C-2}$$

or

$$R_t = R_0e^{-(b+g)t} + \frac{b(Q_0-Q_\infty)}{b+g-a}\left(e^{-at} - e^{-(b+g)t}\right) + \frac{bQ_\infty}{b+g}\left(1 - e^{-(b+g)t}\right). \tag{C-3}$$

$$\frac{R_\infty}{Q_\infty} = \frac{b}{b+g}. \tag{C-4}$$

Fig. 2 (Askovitz). Equations describing transfer from Q to R by diffusion, with continuous proportional outflow from R. (A) Concentration in Q considered constant. (B) Concentration in Q falling exponentially to zero. (C) Concentration in Q varying asymptomatically toward limiting value Q_∞.

by way of several membranes or channels, including both flow or secretion and diffusion mechanisms. Setting down these conditions mathematically leads to new equations which are no more complex than the ones already solved above.

Case 13

Transfer by flow or secretion and diffusion, with proportional outflow from second compartment; "source" (that is, concentration in the first chamber) held constant (fig. 3A).

Case 14

Transfer by flow or secretion and diffusion, with proportional outflow from second compartment: "source" falling exponentially to zero (fig. 3B).

Case 15

Transfer by flow or secretion and diffusion, with proportional outflow from second compartment; "source" approaching exponentially toward a nonzero limiting value (fig. 3C).

The two-compartment analysis presented in detail above may be quite adequate for studying posterior chamber aqueous humor levels, but for some ions normally present in the circulating fluids, discrepancies arose which could not be readily explained. The introduction of a three-compartment system led to better reconcilement between observed figures and hypothetically predicted values.

However, problems still remained in the interpretation of accumulated data on bicarbonate transfer, with the graphs con-

Case 13. $$\frac{dR}{dt} = hQ + b(Q - r) - gR = (b + h)Q_0 - (b + g)R. \quad (A\text{-}4)$$

$$R_t = (R_0 - \frac{b+h}{b+g} Q_0)e^{-(b+g)t} + \frac{b+h}{b+g} Q_0. \quad (A\text{-}5)$$

$$\frac{R_\infty}{Q_0} = \frac{R_\infty}{Q_0} = \frac{b+h}{b+g}. \quad (A\text{-}6)$$

Case 14. $$\frac{dR}{dt} = hQ_t + b(Q_t - R_t) - gR_t = (b+h)Q_0e^{-at} - (b+g)R. \quad (B\text{-}3)$$

$$R_t = R_0e^{-(b+g)t} + \frac{(b+h)Q_0}{b+g-a}(e^{-at} - e^{-(b+g)t}). \quad (B\text{-}4)$$

Case 15. $$\frac{dR}{dt} = hQ_t + b(Q_t - R_t) - gR_t = (b+h)(Q_0 - Q_\infty)e^{-at} + (b+h)Q_\infty - (b+g)R \quad (C\text{-}5)$$

$$R_t = \left[R_0 - \frac{(b+h)(Q_0-Q_\infty)}{b+g-a} - \frac{(b+h)Q_\infty}{b+g}\right]e^{-(b+g)t} + \frac{(b+h)(Q_0-Q_\infty)}{b+g-a}e^{-at} - \frac{b+h}{b+g}Q_\infty, \quad (C\text{-}6)$$

or $$R_t = R_0e^{-(b+g)t} + \frac{(b+h)(Q_0-Q_\infty)}{b+g-a}(e^{-at} - e^{-(b+g)t}) + \frac{b+h}{b+g}Q_\infty(1 - e^{-(b+g)t}) \quad (C\text{-}7)$$

$$\frac{R_\infty}{Q_\infty} = \frac{b+h}{b+g}. \quad (C\text{-}8)$$

Fig. 3 (Askovitz). Equations of transfer from Q to R by both flow (or secretion) and diffusion, with continuous proportional outflow from R. (A) to (C) as in Figure 2.

taining too many inflections to be well fitted even by a double or triple exponential system. It was, therefore, decided to attempt to extend the type of analysis already published so as to make the equations more flexible.

By minimizing special subscripts and Greek letters, and by discussing the essential intermediate steps, it is hoped that the results may be of some value to other investigators in this field.

We may consider the following separate compartments: circulating plasma, cornea, aqueous humor of the anterior chamber, aqueous humor of the posterior chamber, crystalline lens, and vitreous humor. The most general solution would permit any combination of secretion, diffusion, and outflow amongst all pairs of compartments. For many substances, however, a number of simplifying assumptions may be utilized, in order to keep the equations within workable proportions. Thus, for some of the aqueous humor constituents, the lens and vitreous may be considered as an inert body and omitted from the equations.

To illustrate the mathematical technique, let us take an example of a four-compartment system including *G*, the general circulating plasma, *A*, the anterior chamber, *P*, the posterior chamber, and *R*, the "reservoir" representing lens and vitreous, and so forth (fig. 4). Under quite general assumptions, the equations may be reduced to a standard form (fig. 5).

Instead of proceeding to differentiate these equations in order to eliminate the unknown quantities one by one, it would

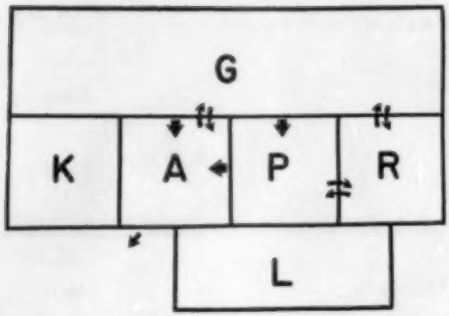

Fig. 4 (Askovitz). Multicompartment system applicable to the eye: (G) General circulating plasma. (K) Cornea. (A) Anterior chamber aqueous humor. (P) Posterior chamber aqueous humor. (L) Crystalline lens. (R) Posterior reservoir of vitreous humor, and so forth. Heavier straight arrows indicate direct flow or secretion; curved arrows, transfer by diffusion; smaller straight arrow, outflow. Numerous other arrangements are possible, including active participation by cornea and lens.

Transfer Equations:

$$\frac{dA}{dt} = c_2 G_1 + c_4 P + c_3 (R - A) - c_5 A \tag{D-1}$$

$$\frac{dP}{dt} = c_6 G_1 + c_8 (R - P) - c_7 P \tag{D-2}$$

$$\frac{dR}{dt} = c_9 (G_1 - R) + c_{10} (P - R) \tag{D-3}$$

Terms Combined:

$$\frac{dA}{dt} = k_1 P + k_2 R + k_3 A + k_4 G_1 \tag{D-4}$$

$$\frac{dP}{dt} = k_5 P + k_6 R + k_7 A + k_8 G_1 \tag{D-5}$$

$$\frac{dR}{dt} = k_9 P + k_{10} R + k_{11} A + k_{12} G_1 \tag{D-6}$$

Circulating Concentration:

$$G_1 = (G_0 - G_\infty) e^{-k_0 t} + G_\infty \tag{D-7}$$

Form of Solution:

$$A = b_1 e^{-k_1 t} + b_2 e^{-k_2 t} + b_3 e^{-k_3 t} + b_4 e^{-k_4 t} + b_5 \tag{D-8}$$

$$P = b_6 e^{-k_1 t} + b_7 e^{-k_2 t} + b_8 e^{-k_3 t} + b_9 e^{-k_4 t} + b_{10} \tag{D-9}$$

$$R = b_{11} e^{-k_1 t} + b_{12} e^{-k_2 t} + b_{13} e^{-k_3 t} + b_{14} e^{-k_4 t} + b_{15} \tag{D-10}$$

Fig. 5 (Askovitz). The equations of transfer for the system illustrated in Figure 4, indicating the general form of the solution to the differential equations.

seem preferable to apply the standard results which are available in textbooks on differential equations.[13, 14]

For a system of simultaneous linear differential equations of the first order, it is known that the solutions consist of sums of several exponential terms. The problem is thus reduced to the determination of the unknown exponents and then the coefficients. For numerical data, this is perhaps best accomplished by means of determinants.

The resulting equations are now being applied to a variety of experimental data to determine whether any new information on aqueous humor dynamics may thereby be obtained.

As far as fitting exponential equations to tabulated data, the following method[15–18] is recommended by mathematical analysts. Let us take a series of experimental values, $y_0, y_1, y_2, y_3, \cdots, y_n, y_{n+1}, \cdots$, assumed to represent the level of some test substance at equally spaced time intervals, and let us attempt to fit the best double exponential curve

$$y = pe^{at} + qe^{bt} + E(t), \tag{27}$$

where the error term $E(t)$ is included to represent the deviations of observed from calculated values.

The exact equation $u = pe^{at}$ has the property that for equal spacing of the time values t, the successive u-values will maintain a constant ratio to each other:

$$u_{n+1} = pe^{a(t+1)} = pe^{at}e^{a} = e^{a}u_n = Au_n. \tag{28}$$

A similar statement applies to $v = qe^{bt}$:

$$v_{n+1} = qe^{b(t+1)} = qe^{bt}e^{b} = e^{b}v_n = Bv_n \tag{29}$$

but the ratio will not be the same. However, we may combine the two into a single *finite difference equation* valid for the sum $w = u + v$:

$$\begin{aligned} w_{n+2} - (A+B)w_{n+1} + ABw_n \\ = u_{n+2} - Au_{n+1} - B(u_{n+1} - Au_n) \\ + v_{n+2} - Bv_{n+1} - A(v_{n+1} - Bv_n) = 0. \end{aligned} \tag{30}$$

For experimental data, such a relationship, that is,

$$y_{n+2} = gy_{n+1} - hy_n, \tag{31}$$

would hardly be expected to be valid for the entire series of observed numbers. However, we may write down these equa-

tions, and obtain the uniquely determined best set of values for the unknown constants g and h by least squares calculations. This leads directly to A and B, and hence to a and b. Having found the exponents, we may apply a similar least squares procedure to work out the coefficients p and q for the curve of best fit of the form given in equation (27).

Summary

The mathematical analysis of aqueous humor dynamics by differential equations has been discussed with several points in mind.

1. The concentration of test substance in the plasma may be taken to vary according to an exponential pattern. This would seem to be preferable for studying data following a single injection of a foreign test substance, rather than to make the assumption that the concentration in the circulation remains constant.

2. The differential equations can be extended to include a lens and/or cornea compartment, and to allow for transfer by flow or secretion or diffusion between every possible pair of compartments.

3. The fitting of numerical equations to experimentally obtained data can be done by an exact least squares process, not subject to the errors which may enter into the graphic methods previously used.

The application of these refinements to the published data of other investigators, and to results recently obtained on aqueous humor bicarbonate levels, is now in progress.

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Acknowledgment

I wish to express my sincere appreciation to Dr. Irving H. Leopold for his encouragement and guidance, and to Dr. Harry Green and Dr. Enrique Wudka for their valuable discussions.

List of principal symbols used in equations (1) to (26) and (A-1) to (C-8)

Q = Concentration of test substance in "source" compartment Q.
R = Concentration of test substance in second compartment R.
Q_0, R_0 = Initial concentrations
Q_e, R_e = Final steady-state or equilibrium concentrations
a = Percentage rate of decay per unit time of the concentration of the test substance in compartment Q.
b = Coefficient of diffusion between chambers Q and R, given as the percentage of excess concentration in Q over R which is transferred from Q into R per unit time.
c, h = Coefficient of flow or secretion out of compartment Q, expressed as percentage of substance in Q transferred out per unit time.
f, g = Coefficient of outflow from chamber R, as percentage of substance in R transferred out per unit time.

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Discussion

Dr. V. Everett Kinsey (Detroit): Dr. Askovitz is to be congratulated for his clear presentation of the fundamental problems involved in the mathematical treatment of data concerning intraocular fluid dynamics.

Since I am not a mathematician nor have I had the privilege of seeing these equations for more than a few minutes at lunch time, I will confine my remarks to two items of general biologic interest.

As Dr. Askovitz has mentioned, there are several difficulties involved in attempting to obtain quantitative results with regard to rate of transfer of substances from the blood to the aqueous humors. The experimenters ordinarily have two choices. One is to inject a test substance or an isotope intraperitoneally or intramuscularly, in which case the concentration in the plasma rises to a maximum over the period of about 20 to 30 minutes. However, as Dr. Askovitz points out, this increasing plasma concentration cannot be entirely ignored with regard to determining how fast the test substance gets into the aqueous humor, either posterior or anterior.

The second choice is to inject it intravenously, in which case there is initially a very high concentration in the plasma and a subsequent decrease. The concentration reaches a plateau which may be flat over a considerable period of time depending on the test substances one is dealing with.

The varying concentration, too, introduces considerable complications in trying to calculate the coefficients of transfer of the plasma to the aqueous humor. Dr. Grant and I faced this problem as long ago as 1942, and wrote a differential equation which described the rate of entrance of the test substance from the peritoneum into the blood. This differential equation then had to be introduced into the general formulations of our hypotheses as to how the substance went from the plasma to the aqueous humor.

As Dr. Askovitz also indicated, the subsequent solution of the equations is both cumbersome and tedious, and when one is finished there still remains a number of uncertainties, one of which is trying to draw a curve on logarithmic paper which best fits the experimental data.

The second point concerns the use of the method of least squares to avoid bias of fitting logarithmic curves. This method is commonly used for curve-fitting and as Dr. Askovitz said is one that is mathematically correct. However, its unrestricted use may lead to another kind of bias. For instance, and this is not uncommon with biologic data, the majority of the experimental data fall close to a line but several points fall far off the line, the method of using least squares will properly take into account the effect of both points. However, this is no guarantee that the line so drawn may not represent the biologic phenomena being studied less well than a curve drawn through the majority of the points, for experimentally induced variations may have been responsible for the wide deviations of the data.

I am looking forward to seeing the application of the mathematical treatment described by Dr. Askovitz to various experimental data on intraocular fluid dynamics.

Dr. Maurice (London): I, too, would like to congratulate Dr. Askovitz on the clarity of his exposition. I am afraid he has made it rather too simple, because on the diagram on the board there is no mention of exchange between the posterior chamber and the vitreous.

The resulting analysis that I have done recently also comes from Friedenwald and Becker's analysis, that the difference between the posterior chamber and the vitreous is greater in the first hour than the change in the vitreous and aqueous humor. Fortunately this exchange does not follow the first equation but follows the second one. I am afraid it is going to make the analysis far more difficult than Dr. Askovitz thinks.

(Slide) I approached this problem from a differ-

ent aspect, as I assumed the change of concentration in the posterior chamber from the results of Kinsey and Palm, and worked out from that the rate of movement from vitreous humor in this case to the anterior chamber, and the rate of accumulation in the posterior chamber itself.

Down here is the time in hours. Up this way is the rate of accumulation of sodium. Here is the change of the eye.

Curve C is the greater increase in concentration of sodium in the posterior chamber. It is also the rate at which sodium moves from the posterior chamber by flow to the anterior chamber.

Curve A is the rate of accumulation of sodium in the posterior chamber itself.

The sum of these three curves is curve D, which is potentially a straight line, and curve E is calculated to be the rate of movement of sodium from the plasma into the posterior chamber, assuming the flow rate calculated by Friedenwald and Becker. Curve D corresponds to the terminal movement from the posterior chamber. It should be the same. You see by calculation they are more or less the same.

This treatment requires a knowledge of concentration in the posterior chamber, and it can be worked out from the rate of change of concentration alone. I wish Dr. Askovitz luck. It is a very difficult problem.

Dr. Langham (London): There is just one question I should like to ask Dr. Askovitz, and that is whether the solution would be much more complicated if one assumes that R on the second equation is not equal throughout the chamber.

In all our kinetics of penetration of the blood aqueous barrier we have to assume there is constant mixing; as the substance comes across the barrier we get an even distribution throughout the aqueous humor, but in point of fact when we have a substance which penetrates the barrier rapidly it will be the time of the mixing.

I wonder whether Dr. Askovitz would comment on this.

Dr. Samuel I. Askovitz (closing): I would like to thank all the discussers for their comments.

In answer to the questions that were asked, as far as an interchange between vitreous and other chambers is concerned, all that this requires is just adding a few more lines on the diagram (fig. 4). The equations are so set up that interchange may be considered between every possible pair of chambers.

I wrote down on the diagram on the blackboard those transfers that seemed more important for actual test substances, but the equations themselves are no more or less complicated by considering the interchange of test substances between every possible pair of compartments.

As far as second order differential equations are concerned, yes, the problems would be more complicated. However, the type of curve shown on the slide, I believe, can be fitted by a sum of first-order exponential terms. This has been done for some of the other available experimental data.

In answer to the question concerning even mixing in a compartment, that is a problem which confronts the cardiac physiologist to quite an extent. As a first approximation, it has been assumed that complete mixing takes place instantaneously. Another way of looking at the equations is to say that each letter represents the average concentration throughout the compartment, even though it is realized that the concentration just at the point of entry of the material from the posterior to the anterior chamber, for example, may be higher than elsewhere in the chamber.

So, we either consider the average concentration or assume that instantaneous mixing does take place. It would considerably complicate the equations to permit a variable concentration throughout the chamber and to make use of that variable concentration in the analysis.

ELECTROMYOGRAPHY OF EXTRAOCULAR MUSCLES IN FUSIONAL MOVEMENTS*

I. Electric phenomena at the breakpoint of fusion

Frederick C. Blodi, M.D., and Maurice W. Van Allen, M.D.
Iowa City, Iowa

Fusional movements have been studied extensively since 1900 when Hofmann and Bielschowsky[1] published their classic paper. They spoke of fusional movements as mediated through psycho-optical reflexes which depend upon the attention of the examinee but cannot voluntarily be initiated nor stopped except for convergence which has a voluntary component. An efficient and complex motor and sensory apparatus is obviously necessary for the adequate and smooth execution of these movements.

Artificially induced fusional movements are an expression of binocular adaptation. They can most accurately be produced and measured by a haploscope such as that introduced by Hering. Prisms are most commonly used to elicit and evaluate such movements. Of cardinal importance is the final breakpoint at which the stimulus to fuse is finally overcome by the increasing disparity of the retinal images. The measurement of the breakpoint in degrees or prism-diopters has become a routine office procedure and its importance is unchallenged. Fregnan[2] has recently summarized our knowledge and theory on this point.

Little work on the other hand has been done to elucidate the neuromuscular mechanisms which underlie the physiologic changes at the breakpoint. The controversy that exists on the question of an active versus a passive divergence could be extended to the fusional movements. The sudden break after an induced maximal convergence could be postulated to be passive and due to a sudden relaxation of the internal rectus muscle or it could be due to an active contraction of the external rectus muscle. A similar, but reverse, mechanism could also explain the sudden break after an artificially induced maximal divergence. Up to the present time all explanations of this mechanism remained mere theoretical speculations. It seemed reasonable to assume that electromyographic studies of the involved muscles might clarify this problem.

Muscle action potentials on congergence have already been studied by Breinin and Moldaver.[3] They found an increased activity of the internal rectus muscle until just prior to the break. Immediately before the break the electrical activity of the external rectus muscle dropped to zero. The breakpoint, however, was not further investigated and no other electromyographic studies on this subject have come to our attention.

Method

Our method followed closely that of Breinin and Moldaver.[3] Concentric needle electrodes were used. They were prepared with enamelled No. 40 copper wire fixed in the lumen of a No. 27 gauge hypodermic needle. The muscle action potentials were amplified through a Grass P4A 4-stage capacity coupled preamplifier. Two channels were used to permit simultaneous observation of two muscle potentials on a DuMont 322-A dual-beam oscilloscope. The traces were photographed on moving paper with the Grass C4D Kymograph camera.

The needles were inserted directly into the extraocular muscle after the instillation of a few drops of 0.5-percent pontocain. No harmful effects of needle insertion were noted.

* From the Ophthalmologic and Neurologic Services of the Veterans Administration Hospital, Iowa City, Iowa.

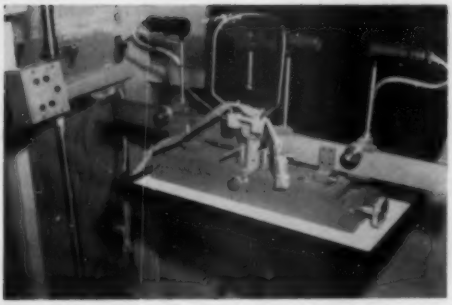

Fig. 1 (Blodi and Van Allen). The haploscope in the shielded room. The signal for the patient lies on the chin rest.

The examinations were done on volunteers who had good vision (without glasses) in both eyes and who had excellent fusional amplitudes with a definite breakpoint. Ten different and independent runs were made on seven subjects.

Fusional movements were elicited with a haploscope put at our disposition by Dr. H. M. Burian. The haploscope was mounted on a table in the shielded room (fig. 1) and adjusted to the patient. Either arm or both arms together were moved. When the activity of only one muscle was recorded the second channel was used for a signal given by the patient pressing a button to indicate diplopia or fusion. This signal interrupted the 60-cycle sine wave used as an indicator of the speed of paper movement. The prism tests were done while the patient was fixing at near or at distance.

Results

1. Convergence on the haploscope

A. *The external rectus muscle* shows a slow decrease in its action potentials from looking straight ahead until maximal convergence has been reached. There is at the same time an apparent decrease in frquency of motor unit activity (fig. 2). When the breakpoint is being approached the tracing becomes somewhat irregular due to movements of the eyeball. In a few subjects these movements are so marked that the beam of

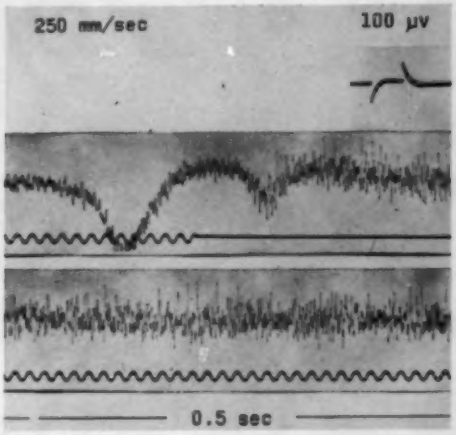

Fig. 2 (Blodi and Van Allen). The upper tracing represents the electrical potential of the external rectus muscle at the breakpoint. The lower tracing represents the muscle when looking straight ahead.

the oscilloscope shifted off the face of the tube (fig. 3).

When the breakpoint is reached the electrical activity of the external rectus muscle picks up suddenly and vehemently (fig. 4). Before the breakpoint is reached the average potential of motor unit activity of the external rectus muscle decreases usually to less than 100 μV. At the breakpoint this potential increases by a factor which is never less than 2.2 and may be as high as 26. The average value of this factor based on the measurement of the potentials in 12 inde-

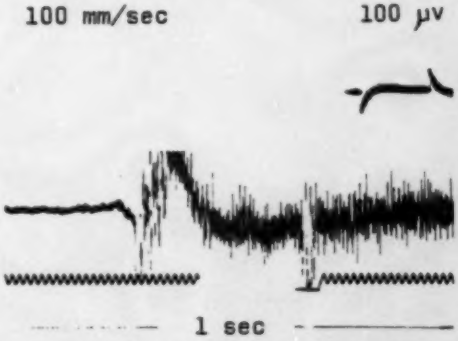

Fig. 3 (Blodi and Van Allen). The lateral rectus muscle at the breakpoint. The patient's signal indicating diplopia interrupts the sine curve.

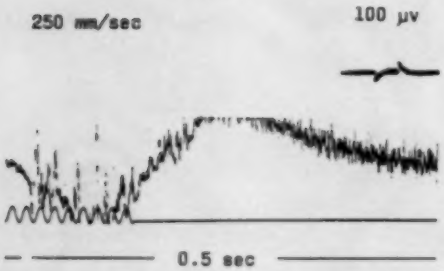

Fig. 4 (Blodi and Van Allen). The lateral rectus muscle at the breakpoint with some large motor units.

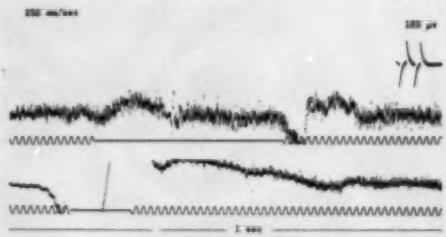

Fig. 6 (Blodi and Van Allen). Upper tracing: no change in potential of the external rectus in asymmetric convergence when the tested eye does not move. Lower tracing: external rectus potential in normal convergence.

pendent runs was 6.2. The absolute values mean here very little as they depend so much on needle placement. The frequency of motor unit activity increases at the breakpoint and the signal given by the patient when he first sees double usually follows the electric phenomena, the lag being due to the reaction time of the individual. The movement of the eye when convergence is decreased and the patient fuses again is associated with a shift of the tracing but with no change in the electric potentials (fig. 5).

The changes at the breakpoint are quite similar whether the convergence is symmetrical or whether only the tested eye moves. If the tested eye does not move because of asymmetric convergence of the other eye usually no change in the potentials can be discerned (fig. 6). In one instance, however, there appeared to be a slight increase (from 60 to 80 μV) in the activity of the external rectus muscle at the breakpoint even when only the arm in front of the not-tested eye was moved (fig. 7).

B. *The internal rectus muscle* shows on convergence an increase in electric potentials and an increase in frequency. Here again as the breakpoint is approached a few nystagmoid movements become manifest. The near-point of convergence and the breakpoint itself are characterized by a sudden decrease of the potentials and the frequency of the motor units (fig. 8A). These potentials decrease by a factor of at least two and averaged in six independent runs 4.2. No change in the electric activity could be detected in asymmetric convergence when only the non-tested eye moved (fig. 8B).

C. *When both horizontal muscles* of one eye are tested the changes at the breakpoint occur simultaneously (fig. 9). In one in-

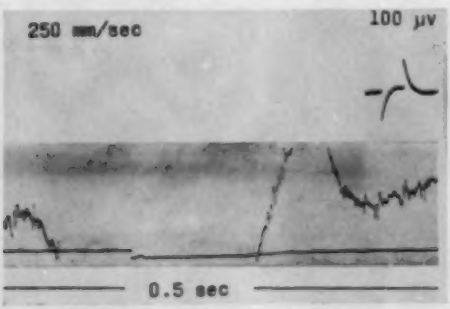

Fig. 5 (Blodi and Van Allen). Shift of the entire tracing of the external rectus potential with movement of redress.

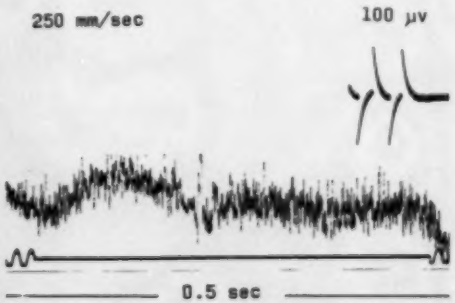

Fig. 7 (Blodi and Van Allen). Slight increase in the potential of the external rectus in asymmetric convergence when the tested eye does not move.

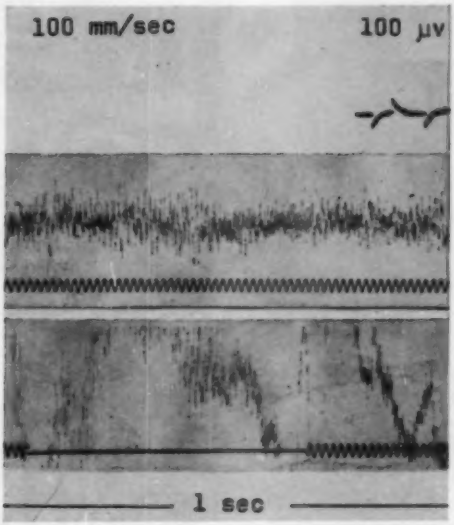

Fig. 8A (Blodi and Van Allen). The upper tracing is from the internal rectus muscle with the eye looking straight ahead. The lower tracing is from the internal rectus muscle at the breakpoint of convergence.

stance we had the impression that the decreased activity of the internal and the increased activity of the external rectus muscle preceded the actual breakpoint by less than 0.2 seconds (fig. 10). This change in activity was a gradual one which led into the abrupt change at the breakpoint.

2. Divergence on the haploscope

Divergence up to 10 prism diopters did not produce any appreciable change in the activity of the external rectus muscle (fig. 11). The movements of the eye at the breakpoint were nevertheless recognizable because of the shift in the entire tracing. The same held true for the internal rectus muscle (fig. 12) in which the electric potential did not change during divergence or at the breakpoint.

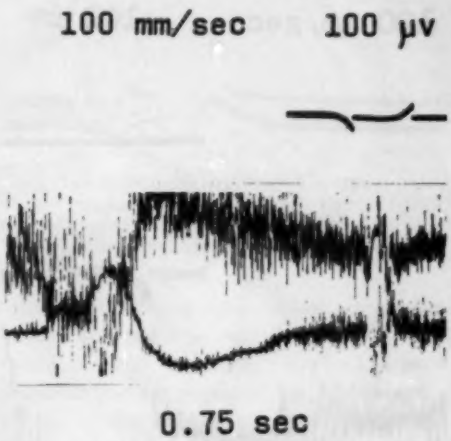

Fig. 9 (Blodi and Van Allen). Simultaneous tracing of both horizontal muscles at the breakpoint of convergence. The upper tracing is from the external, the lower tracing from the internal rectus muscle.

3. Fusional movements with prisms

Generally speaking the electrical responses associated with convergence and divergence when elicited with rotating prisms resemble those produced by the haploscope. The ex-

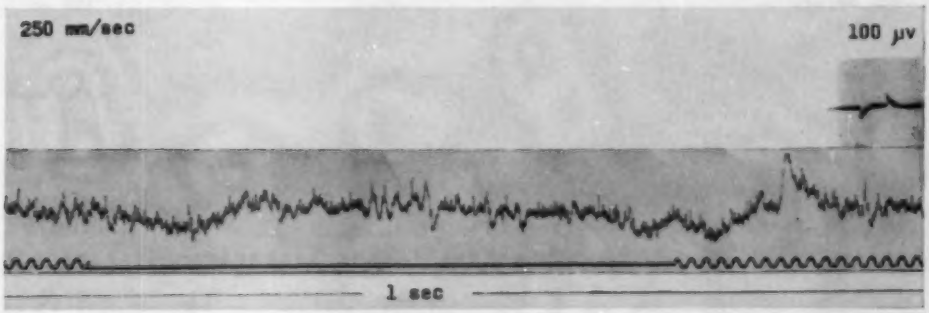

Fig. 8B (Blodi and Van Allen). The potential of the internal rectus muscle in asymmetric convergence when the tested eye does not move.

Fig. 10 (Blodi and Van Allen). Changes in the potential of the internal rectus (upper tracing) and the external rectus (lower tracing) just preceding the breakpoint of convergence.

cursion of the prism has to be of a considerable magnitude before an electric change is appreciated. A base-out prism of 10 diopters, for example, did not produce any change in potentials (fig. 13). This held also

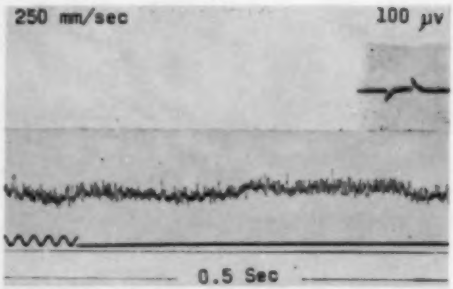

Fig. 12 (Blodi and Van Allen). The potential of the internal rectus muscle at the breakpoint of divergence.

for base-in prisms as would be expected from our experience on the haploscope (fig. 14).

Discussion

The most important finding of our experiment is the fact that an active contraction of the external rectus muscle occurs at the breakpoint of convergence. This is associated with a simultaneous relaxation of the internal rectus muscle. These processes go on regardless of the mode by which the vergence is obtained. Most of our experiments were done on the haploscope, but identical results were found with rotating prisms and with convergence beyond the near-point. This phenomenon is open to theoretic interpretations.

The fact that an active innervation of the lateral rectus muscles occurs at the break-

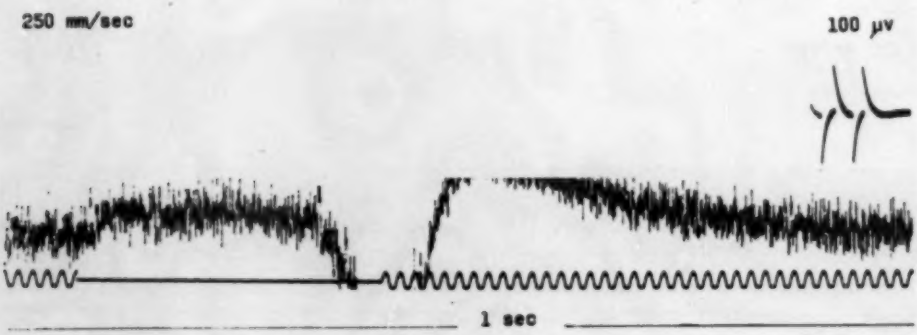

Fig. 11 (Blodi and Van Allen). The potential of the external rectus muscle as the breakpoint of divergence.

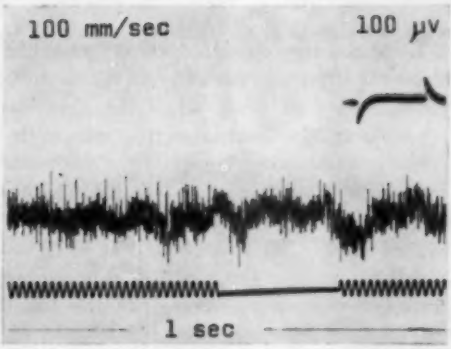

Fig. 13 (Blodi and Van Allen). The external rectus muscle at the breakpoint of convergence with a 10-diopter base-out prism.

point could be another proof for an active divergence mechanism. It certainly speaks against a mere cessation of the activity of the internal rectus muscles with passive return to a "position of rest" when the fusional amplitude for convergence has been exhausted.

Most attractive to us seems the theory that the eye is normally in a delicate and well-regulated equilibrium as far as the innervation of both horizontal muscles is concerned. We could assume that the sum total of innervation transmitted to the horizontal agonist and the antagonist remains the same regardless of the position of the eye, guaranteeing a perfectly balanced movement at all times.

An increased electrical activity will occur in the external rectus muscle whenever the

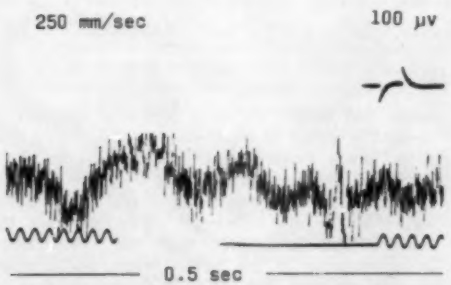

Fig. 14 (Blodi and Van Allen). The external rectus muscle at the breakpoint with a six-diopter base-in prism.

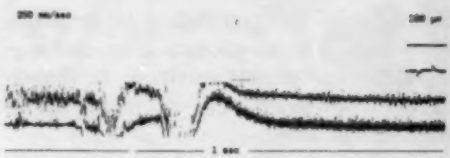

Fig. 15 (Blodi and Van Allen). The lateral rectus (lower tracing) and the medial rectus (upper tracing) at the breakpoint of convergence in a patient with intermittent exotropia.

activity of the internal rectus muscle decreases and such a reciprocal activity between the two horizontal muscles has been demonstrated for versions[3] and for the optokinetic nystagmus.[5] The behavior of the internal rectus muscle is indeed absolutely complementary and the changes in its electrical activity occur simultaneously with the changes in the external rectus muscle. In no case, not even in a patient with intermittent exotropia, could we find an increased activity of the lateral rectus muscle before the inhibition of the internal rectus at the breakpoint occurred (fig. 16). This is in contrast to the findings of other authors.[3,6]

It could be assumed that the stretch reflex postulated by Breinin[4] would suddenly lead to an increased activity of the external rectus muscle and account for the break. Breinin assumed the presence of such a reflex on the basis of two findings. One was the disappearance of all electrical activity when during an enucleation the muscle was severed and allowed to retract into the orbit. Electric

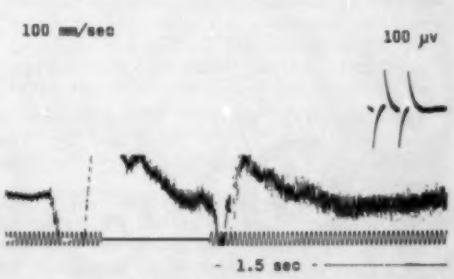

Fig. 16 (Blodi and Van Allen). The external rectus muscle when convergence is interrupted on command.

activity could be again elicited by stretching the muscle. The second finding was the augmentation of electrical activity in a few paretic muscles when the eye was moved out of their field of action.

This stretch reflex may play a role here (as contrasted with versions) because both external rectus muscles are stretched and the effect may be additive. The fact that fusional convergence may be increased with exercises would speak against this theory. In addition, exactly the same tracings can be obtained when convergence is interrupted on command (fig. 15). This voluntary break can certainly not be caused by a proprioceptive mechanism.

The breakpoint after a fusional divergence movement did not lead to any change in the electric pattern of either horizontal muscle detectable by our method. The small divergence amplitudes do not carry the eye beyond the range in which a decrease or increase in electric activity can be measured at the present time.

Summary

1. At the breakpoint of convergence the electrical activity of the external rectus muscle increases suddenly while the electrical potential of the internal rectus muscle decreases. This occurs regardless of the mode by which convergence is elicited (haploscope, prisms, near vision).

2. There is no change in the electrical potentials of the horizontal muscles when during asymmetric convergence the tested eye does not move.

3. The breakpoint of divergence did not produce any change in the electrical activity of the tested muscles under the conditions of our experiment.

4. The theory is presented that the sum total of innervation to the two horizontal muscles has to remain the same regardless of the position of the eye.

University Hospitals.

We wish to express our appreciation to Dr. H. M. Burian for his guidance and criticism. Without his help this work could never have been done.

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Discussion

Dr. Goodwin M. Breinin: It is a pleasure to discuss this paper by Dr. Blodi and Dr. Van Allen because it is an important contribution to an important topic.

Just to set the historical record straight, I think in the original publication on this subject several years ago we described the divergence mechanism with a clear-cut description of the breakpoint, the divergence activity, and so on.

However, the observations which Dr. Blodi reports are essentially the same as we have found. We have also carried out studies in fusional movements and studies on many forms of vergence. I thought it would be interesting to show you a few slides which depict characteristic situations.

[Slide] This is approximation vergence with the near point of convergence. The lateral rectus decrements as the eye converges. Here we have the characteristic breakpoint with the synchronized large discharge of the lateral rectus, and here an even larger one. This break is exactly the type of thing that the author described. It is of interest that it is of this large synchronized type.

[Slide] This is prism vergence, and here again

we see a lateral rectus which decrements. The integrator trace permits exact quantitation of innervation. All one need do is to measure the vertical level of the integrator discharge to get the integral of the energy developed within the given time intervals.

We see that the energy levels decrement up to convergence and then there is the big burst which is charactristic of the breakpoint, and then the larger firing of the eye as it diverges.

Here we have prism convergence with the prism before one eye. The innervation of the other eye in this case stays constant. I should put it this way: We are recording from the eye which does not have the prism in front of it, and which therefore remains still and shows no change in its innervation.

This is accommodative convergence. Here is the trace of a medial rectus when a minus lens has been placed in front of the other eye, producing accommodative convergence with increased firing.

[Slide] This is the situation in which the minus lens is placed before the fixing eye, and in this case there is no change in the innervation of the fixing eye. This is again an important feature.

[Slide] This is the constant level when the lens is before the eye that does not move. In other words, when an eye does not move there is no increase in innervation. When an eye moves there is an increase of innervation. When we put minus lenses in front of both eyes there is no increase of innervation within the limits of the relative fusional divergence. Only when we exceed the relative fusional divergence is there a break, and then the eye that turns in shows the expected increase of innervation. The eye that does not turn in shows no change.

The mechanism that Dr. Blodi and Dr. Van Allen have described and that we have reported shows that there is integrated in the brain all forms of vergence and version. These are added together, and what emerges into the final common path is the vector resultant.

This is a simple reciprocity mechanism in which there is never any co-contraction. A medial rectus does not compete in a tug of war with a lateral rectus to hold the eye in position. They are always associated in this simple reciprocity relationship.

Divergence is an active process. We have recorded divergence in many ways. One of the slides showed fusional divergence on the amblyoscope, and it shows the increase in divergence when one arm of the instrument is diverged. Using the integrator it is possible to evaluate and to quantitate innervation that is not obvious to the naked eye by simple inspection methods.

To summarize, then, the forms of innervation are all integrated in the brain and emerge as a simple reciprocity mechanism. I would take issue with Dr. Blodi's statement that the innervation to the extraocular muscle is always constant, that is, the sum of the innervation of antagonists is the same. Actually, he is really describing the reciprocity relationship. We have evidence which shows that under certain conditions the sum of the levels of innervation of antagonists may be widely disparate. But in essentials I am sure we agree quite closely, and we are all looking forward with interest to further studies on this subject.

DR. HERMANN M. BURIAN (Iowa City, Iowa): The paper by Dr. Blodi and Dr. Van Allen adds a new and exciting chapter to the growing story of electromyography of the extraocular muscles. Their findings shed light on one of the most important processes in the physiology of extraocular motility.

It has been my contention for many years that fusion is the result of an equilibrium between the forces which tend to keep the eyes properly aligned and those forces which tend to disrupt the alignment of the eyes. The most powerful, though not the only stimuli which contribute to keeping the eyes so positioned that sensory fusion is possible are the stimuli arising from the retinas. It is largely due to them that we have single binocular vision.

These stimuli would evoke such motor impulses that a balanced tonus is created in the extraocular muscle. Because of this I have frequently objected to such terms as "muscle imbalance" in the definition of heterophoria and to the definition of orthophoria as a condition in which the extraocular muscles are "in balance." The tonus of the extraocular muscles is always in balance, even in comitant strabismus, except for frank paralyses.

These were theoretical deductions, however. Evidence that these deductions were correct is coming now from the electromyographic studies of Dr. Blodi and Dr. Van Allen, and of others. These studies have many implications not only for the physiology of the extraocular muscles, but also for our clinical work. I have often emphasized that the recession of a muscle affects the action of the antagonist as well as that of the recessed muscle itself, not only through purely mechanical changes in their relationship, but also through adjustments in the impulses flowing to them. This assumption has been made even more probable. It is my hope that it may sometime be tested directly by electromyography.

The most important finding in the study is the tremendous increase in the potentials of the lateral rectus muscle when the breakpoint of induced convergence is reached. This finding, which has its parallel in the increased activity of the lateral rectus muscle when a patient with intermittent exotropia goes from fusion to exotropia, may at first seem disturbing to some of us. On reflection, however, the observed facts appear to be reasonable. The stimulus resulting from the increasing disparity of the retinal images, when convergence is forced, produces an ever growing increase in the impulses to the medial rectus muscles with corresponding reduction in the impulses to the lateral rectus muscles. When the maximum effective disparity is exceeded, the flow of impulses to the medial rectus muscles stops suddenly and the lateral rectus muscles receive powerful motor im-

pulses, maintaining the balance of tonus between these antagonists. This does not necessarily imply the existence of a "divergence center" which should presumably be effective mostly in absolute diveregence, that is, in bringing the eyes into a divergent relative position beyond parallelisms of the visual lines. Nor does it necessarily imply that the tonus of the extraocular muscles is regulated through proprioceptive mechanisms. It does imply that there are central nervous arrangements which finely regulate and integrate the flow of impulses to the extraocular muscles and preserve that balance in the tonus of these muscles which is so essential to the smooth and precise execution of the eye movements.

I wish to congratulate Dr. Blodi and Dr. Van Allen on their most interesting presentation. I look forward eagerly to the further advances which they, and other workers in the field of electromyography, will contribute toward the understanding of the physiology and pathology of the extraocular muscles.

DR. FRANCIS H. ADLER (Philadelphia): I can well understand Dr. Blodi's hesitancy in deriving any certain knowledge of a divergence mechanism from his experimental data. Up until the advent of electromyography we have been in an argument between two opposing groups, one of whom has stated that divergence was entirely a matter of diminishing convergence with the elastic pull of the lateral rectus muscles and the fascia in the orbit bringing the eyes out to parallelism.

Once and for all, I am just a clinician, and once and for all I would like to lay the ghost of that theory and ask Dr. Blodi if he does not believe that at least these records of his, and those of Dr. Breinin, and my own very poor records which were early obtained in this field, definitely prove that we are dealing not only with a relaxation of convergence but with an active increase in tonus to both lateral rectus muscles.

DR. FREDERICK C. BLODI (Iowa City): I wish to thank the discussers, and I agree with Dr. Adler.

STUDIES ON THE ROLE OF SYMPATHETIC NERVOUS STIMULATION IN EXTRAOCULAR MUSCLE MOVEMENTS*

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Columbus, Ohio

INTRODUCTION

Evidence has been accumulated in recent years that ocular movements can be divided functionally into two types (A) conjunctival movements which are volitional, fast, saccadic, yoked movements of tetanic character obeying Hering's law of equal innervation to the muscles of both eyes, and (B) "follow" or "gliding" movements which are reflexly innervated, slow, unyoked movements of tonic character. Of the latter type are the fixation, fusion, vergence, and slow phase nystagmic movements.[1-5]

There is at present general agreement that the volitional movements are approximately 10 times as fast as the reflex movements.†[1,4,5] However, the nature of the neuromuscular mechanisms of the two types of ocular movements is quite controversial. Numerous attempts have been made to correlate the functional differences with different morphologic substrates. Two theories have been advanced to explain the rapid and slow eye movements. Both views are based mainly on evidence derived from the histologic structure of the external eye muscles:

1. Schubert has suggested that the tetanic (volitional) and tonic (reflex) movements are mediated by two types of striated muscle fibers innervated by two different types of somatic nerve fibers.[1,6,7] This view is supported by older histologic findings of Woollard[8] and by recent work of Krüger.[6,7] The latter distinguishes two kinds of striated muscle fibers, one having a "fibril structure" and the other a "field structure." The "fibril structure" muscle fibers, innervated by thick medullated somatic nerve fibers with end plates, are believed to mediate tetanic volitional movements. On the other hand the

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† The velocity of voluntarily initiated convergence movements is apparently also of the same order of magnitude as that of saccadic movements, according to recent unpublished findings of Dr. Gerald Westheimer, School of Optometry, Ohio State University (by personal communication).

"field structure" muscle fibers, innervated by thin nerve fibers with grape-like endings, are believed to be responsible for the tonic (reflex) movements.

2. Alpern and Wolter[9] recently advanced another theory in which they suggest that the final common paths for saccadic (fast) movements are formed by thick somatic nerve fibers, whereas the final common paths for vergence (slow) movements are formed by thin autonomic nerve fibers. This view is mainly based on the histologic investigations of Boeke,[10] Kirsche,[11] and Wolter,[12,13] According to these authors the striated muscle fibers have a double innervation, motor and autonomic. According to Wolter[12] the autonomic fibers can be again subdivided histologically into two types, which are assumed to be sympathetic and parasympathetic in origin.

Since the histologic evidence is partly conflicting it is difficult to assess from this evidence alone which structures represent the morphologic correlates for the slow (reflex) ocular movements, that is, whether these are mediated by the specific "tonic" striated muscle fibers[6,7] or small striated muscle fibers[8] or by the doubly innervated (somatic plus autonomic) striated muscle fibers.[10–13] It appears that physiologic information as to the muscle responses may aid in the elucidation of this problem.

When Alpern and Ellen[4,5] advanced the view that vergence (slow) movements of the eye might be attributed to autonomic activity, one of the discussants (Brecher) expressed caution concerning their hypothesis in the absence of support from conclusive physiologic experiments. Since such experiments have not been reported as yet, it is the purpose of the present and another later study to investigate physiologically whether or not autonomic nervous stimulation causes movements of the external eye muscles or affects the height of their contraction.

Of the two divisions of the autonomic nervous system, only the sympathetic can be distinctly separated anatomically from the somatic nerve supply of the extraocular muscles. This permits a direct experimental approach to the problem. Accordingly, the present paper will deal only with the effects of sympathetic stimulation on the external eye muscles.

Method

Acute experiments were undertaken in 20 cats, 3.0 to 5.0 kg. in weight, anesthetized intraperitoneally with 30 mg./kg. of sodium pentobarbital. Cats were found to be most suitable for this work since their sympathetic and parasympathetic nerves are clearly separable in the upper neck region for stimulation purposes. In dogs these structures cannot be distinctly defined by dissection. The experimental arrangement is illustrated in Figure 1.

The superior cervical ganglion was dissected free from the surrounding tissues and a shielded electrode was applied. This ganglion was stimulated with a Grass stimulator at 25 to 100 cps which induced marked pupillary dilatation and nictating membrane contraction. After the removal of the zygomatic bone and enucleation of the eye, the external eye muscles and nictating membrane were dissected free from surrounding tissue.

Enucleation of the eye and proper isolation of the external eye muscles was necessary in order to permit recording of the muscle contractions, unimpeded by artefacts from movements of surrounding tissues which may also respond to sympathetic

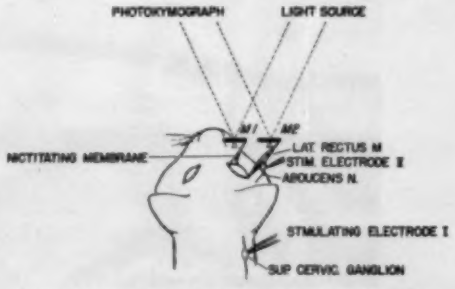

Fig. 1 (Brecher and Mitchell). Schematic representation of the experimental arrangement for determining the effects of sympathetic stimulation on the lateral rectus muscle. Description in text.

stimulation. Great care was taken to assure an unimpaired blood and nerve supply to the eye muscles and to the nictating membrane. This is important because the sympathetic nerves accompany the blood vessels.

The insertion of one of the external eye muscles (for example, lateral rectus) and the tip of the nictating membrane were attached to isometric muscle levers of adequate frequency response and sensitivity. With this arrangement changes in the degree of muscle tension (referred to as "contraction height") could be recorded. Each lever actuated a mirror (M_1 and M_2) for optical recording of the muscle movements. In some experiments (Section B of "Results") a direct stimulation of the abducens nerve was made with a second stimulator, in addition to the superior cervical ganglion stimulation.

Results

A. Mechanical response of extraocular muscles to sympathetic stimulation

Figure 2 illustrates the effect of sympathetic stimulation on the nictating membrane and lateral rectus muscle. The beginning and end of the stimulation of the superior cervical ganglion is marked by X and Y respectively. About 600 msec. after the beginning of the stimulation, the nictating membrane contracted as indicated by a rise in the upper tracing. Relaxation began about three seconds after the cessation of stimulation. During sympathetic stimulation the lateral rectus muscle showed no movements, as indicated by the lack of any deviation from the straight line course of the tracing (second from top). Similar records were obtained in all 10 experiments undertaken with the lateral rectus and the other extraocular muscles.

From this one may conclude that sympathetic stimulation causes neither a contraction nor a relaxation of the external eye muscles.

In order to compare these findings with those obtainable under corresponding conditions in other body regions, the sympathetic supply to the cat hind-limbs was stimulated in four experiments. This did not result in any movement of the skeletal muscles either.

B. Influence of sympathetic stimulation on the height of contraction of extraocular muscles

Although sympathetic impulses do not directly result in extraocular muscle movements, such impulses might, nevertheless, influence the height of extraocular muscle

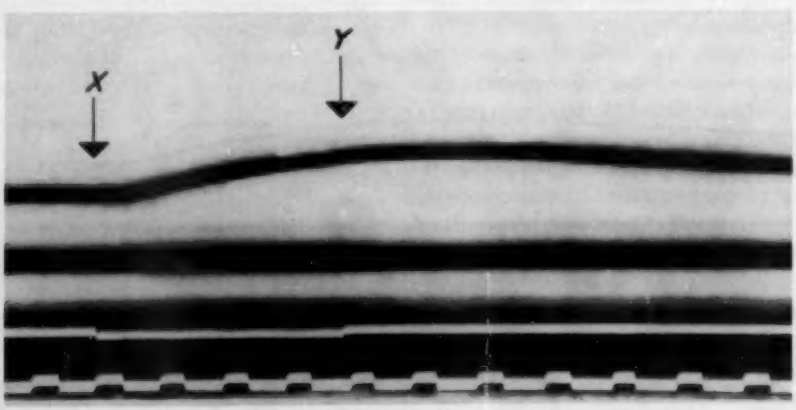

Fig. 2 (Brecher and Mitchell). Contraction of the nictating membrane resulting from superior cervical ganglion stimulation with concomitant recording of lateral rectus muscle tonus. Tracings from top to bottom: nictating membrane, lateral rectus muscle, stimulus marker, time (one second from one elevation to the next). Segment of an original record from a representative experiment. Description in text.

contractions elicited by somatic stimulation. This general problem, concerning the effect of sympathetic stimulation on the height of skeletal muscle contractions, has been studied in the past. Orbeli[14] was the first to observe that the height of skeletal muscle contractions could be augmented by concomitant stimulation of the sympathetic supply to the muscle involved. This so-called Orbeli phenomenon was further investigated by Baetjer[15] who also extensively reviewed the literature. In repeating Orbeli's work Baetjer reported that the experimental results were inconsistent. She found, for example, in some experiments an increase, but in others a decrease or no effect of sympathetic stimulation on the contraction height. These observations were made on skeletal muscles in the hind limbs of frogs (Orbeli) and cats (Baetjer). No investigations seem to have been reported concerning the skeletal musculature of the head.

Experiments of the Orbeli type were therefore performed in order to study the effect of sympathetic impulses on the height of extraocular muscle contraction.

The results of a typical experiment are illustrated by two segments taken from one

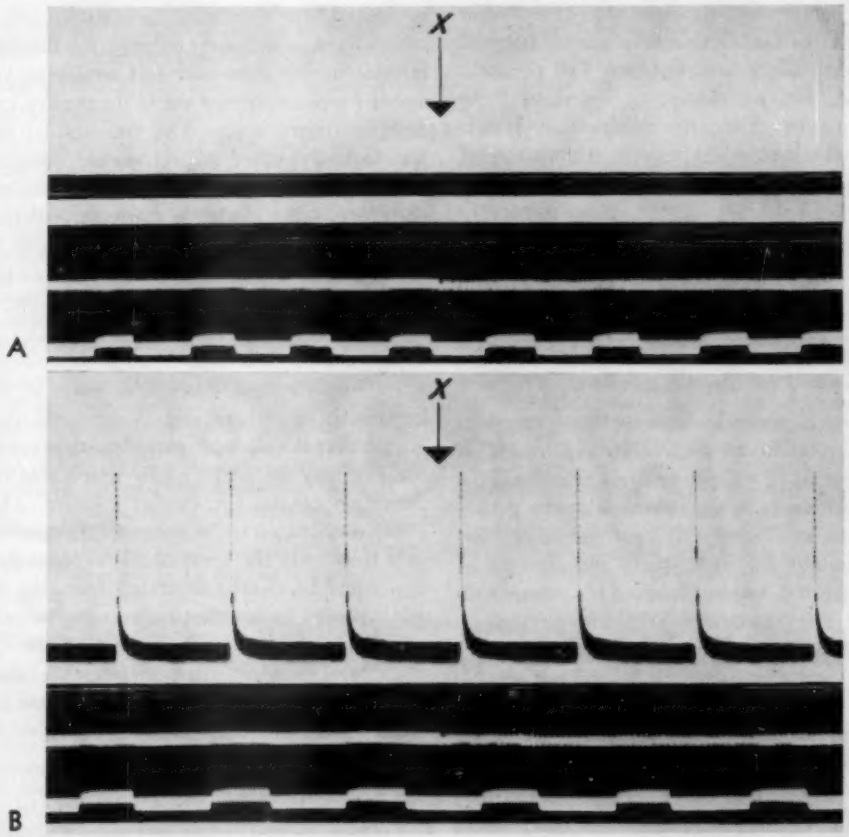

Fig. 3 (Brecher and Mitchell). Effect of superior cervical ganglion stimulation upon the contraction height of the lateral rectus muscle. (A) Control. Tonus of lateral rectus muscle during such stimulation. (B) Contractions of lateral rectus muscle before and during sympathetic stimulation. Tracings from top to bottom: lateral rectus muscle, stimulus marker, time as in Figure 2. Segments of an original record from a representative experiment. Description in text.

continuous record (fig. 3). Segment A is the control in which only the superior cervical ganglion was stimulated (stimulator I in fig. 1). The beginning of the stimulation is marked X. The tracing of the lateral rectus muscle shows no deflection. In segment B a rhythmic contraction of the lateral rectus muscle is shown when single shocks are applied to the abducens nerve every 850 msec. (stimulator II in fig. 1). At the place marked X the superior cervical ganglion was stimulated concomitantly (stimulator II in fig. 1). One can see that the height of the contractions remained the same.

A total of 10 Orbeli type experiments were performed. A slight decrease (eight percent) of contraction height was observed in two, a slight augmentation (10 percent) in one, and no change in seven of these experiments. Similarly inconsistent results were obtained when tetanic stimulation of the skeletal muscle was used instead of single shocks.

From these results it may be concluded that sympathetic impulses do not affect in a consistent manner the height of extraocular muscle contractions elicited by somatic nerve stimulation.

Discussion

These experiments indicate that sympathetic stimulation as such does not result in movements of the external eye muscles. This observation is in agreement with the generally accepted concept that autonomic nervous stimulation does not cause contractions of the skeletal musculature. This concept is based on substantial previous physiologic evidence. The experimental findings, therefore, do not support the hypothesis of Alpern and Wolter[9] that the vergence (slow) movements of the eye muscles are caused by autonomic innervation. Likewise there is no consistently reproducible effect of sympathetic stimulation on the already contracting eye muscle (Orbeli type experiment).

In preliminary experiments with parasympathetic nerve stimulation no external eye muscle movements could be demonstrated either. However, due to the complex anatomic situation an indirect approach had to be used for separating the effects of parasympathetic and somatic stimulation. This will be reported at a later date.

In order to approach under strictly controlled experimental conditions the problem of the genesis of the slow and fast eye movements it was, of course, necessary to limit the aspects of the problem to one facet only. Since the results concerning the facet investigated here were entirely negative, other possibilities, which may account for the difference in the slow and fast ocular movements (such as pointed out in the beginning) deserve future study. The function of the histologically observed autonomic innervation of the extraocular muscles remains, therefore, still a matter of conjecture. Whatever the function is, it does not appear to be that of eye muscle movement, as far as the sympathetic system is concerned.

Summary

An experimental study was made of the hypothesis of Alpern and Wolter who suggested that the slow vergence movements of the eye may be mediated by autonomic innervation of the extraocular muscles. This study was limited to the sympathetic innervation since only the sympathetic nervous system could be clearly separated from the somatic nerves. In anesthetized cats sympathetic stimulation caused no movement of the eye muscles. Likewise, sympathetic stimulation did not consistently increase or decrease the tension of extraocular muscles produced by somatic nerve stimulation.

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Discussion

Dr. Francis Heed Adler (Philadelphia): To test Alpern and Ellen's hypothesis that the slow movements of convergence are due to autonomic activity, Dr. Brecher and Dr. Mitchell have singled out the sympathetic side of the autonomic supply to the eye muscles and found that sympathetic stimulation did not cause any contraction of the ocular muscles in cats, nor did it change the type of contraction induced by stimulation of the somatic nerves to the muscles. They have therefore eliminated the sympathetic supply from consideration.

Let me point out that their results apply only to cats, and not necessarily to man or other experimental animals. For some unknown reason there is great variability in the anatomic structure and physiologic behavior of the ocular muscles in different species. Cats have no muscle spindles and McCouch and I (McCouch, G. P., and Adler, F. H.: Extraocular reflexes. Am. J. Physiol., **100**: 78, 1932) were unable to demonstrate any stretch reflexes from these muscles. Man and some other animals on the other hand do have spindles of a sort, and show stretch reflexes and proprioception, as recently proved by Breinin electromyographically.

As to the parasympathetic side of the autonomic nervous system the authors state that preliminary experiments failed to reveal any effect on the ocular muscles, but they are going to make this the subject of a separate report. Parasympathetic innervation of the ocular muscles was claimed by Sunaga in 1927 (quoted by Kuntz, A.: Autonomic Nervous System. Philadelphia, Lea & Febiger, 1945, ed. 3, pp. 361) on the basis of histologic changes observed in these muscles following parasympathectomy.

There is good clinical evidence for the existence of two separate mechanisms innervating the medial rectus muscles. Patients with a lesion in the medial longitudinal bundle between the third and sixth nerve nuclei characteristically show failure of adduction of one eye in versions to the opposite side, suggesting a paralysis of the medial rectus muscle, but with retention of good convergence, proving that the medial rectus muscle is actually not paralyzed. The eye cannot be moved by the medial rectus muscle in versions and yet it can in vergences. This is added evidence to that adduced from the difference in speed of versions and vergences under physiologic conditions that the medial rectus muscle is innervated by two separate mechanisms, one of which may be caught in lesions of the medial longitudinal bundle while the other escapes.

Further, in lesions close to the superior colliculi convergence is lost with retention of versions. At the same time the pupils in these patients show changes indicative of parasympathetic damage. This condition is known as Parinaud's syndrome.

On the basis of this clinical evidence, I will hazard a guess that Alpern and Ellen's hypothesis is probably correct; namely, that versions are carried out by somatic innervation while vergences are due to the innervation by the parasympathetic side of the autonomic nervous system.

This does not necessarily mean that there are separate muscle fibers or separate nerve fibers in the final common pathways subserving these two separate innervations, which may only be separate higher up in the neuromuscular apparatus.

Dr. Brecher and Dr. Mitchell have proved their point in a most admirable series of experiments, and I will be interested to hear their final report on parasympathetic stimulation.

Dr. J. Reimer Wolter (Ann Arbor): I would

like to demonstrate some histologic slides which represent examples for the motor, sensory, and double autonomic innervation of the striated fibers of the human eye muscles. These findings were published before and it was suggested that the two types of delicate nerve fibers found on the muscle fibers in addition to the motor end-plates might be sympathetic and parasympathetic in nature. Together with Alpern we suggested that these histologic facts of a motor and autonomic innervation of the eye muscles could be used to explain the difference in vergence and saccadic eye movements.

Dr. Brecher demonstrated in his excellent paper that there is no reaction of eye muscle fibers following electrical irritation of sympathetic centers in the cat. We would like to emphasize that there is a difference between the function of human eye muscles and those of the cat. Furthermore, it is quite possible that the sympathetic nerves of the human eye muscles have only trophic but no motor functions while the parasympathetic system seems more likely to have to do with the slow eye muscle movements.

This study of Dr. Mitchell and Dr. Brecher is a very important contribution. But it answers only half of the question whether there is an autonomic part in the function of human eye muscles.

DR. GOODWIN M. BREININ (New York): The question brought up by the authors about an autonomic innervation is extremely important. We have been exercised by that thought also; and thus far, using various autonomic drugs topically, we can see no difference in the firing of the intraocular muscles. Of course certain drugs do elicit muscle contraction, for example succinylcholine.

Secondly, in the vergences as compared with the versions, I have not yet been able to distinguish any characteristics which would serve to indicate a separate mechanism. I do think that there are different sources of tonus in the extraocular muscles. It comes from many different places, and I believe we have separately isolated an upper and a lower motor neuron mechanism for tonus.

This is a rather complicated topic. Tonus is not a mechanism which I could ascribe to the autonomic system at present.

DR. GERHARD A. BRECHER (closing): I must state that it is always less satisfying to obtain negative results to a problem than positive ones. However, one must accept the results of investigations as answers under the given experimental conditions. In the case of our studies these conditions were controlled to the best of our ability. The negative answer applies, therefore, only to the conditions in which the peripheral outflow of the sympathetic nervous system was tested.

THE EFFECT OF ULTRASONIC RADIATION UPON THE RATE OF ABSORPTION OF BLOOD FROM THE VITREOUS*

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I. INTRODUCTION

This is a preliminary report of attempts at clearing the vitreous of injected autogenous blood by ultrasonic radiation. Numerous reports of the absorption of hemorrhages and exudates from human eyes, following ultrasonic radiation, have appeared in the European literature.[1-11] Since hemorrhages and exudates will frequently absorb without any treatment, clinical evaluation of this method of treatment would require a statistically significant number of cases or appraisal in experimental animals. The results of the latter approach are contained in this paper.

So few papers on ultrasonics have appeared in the American ophthalmic literature that a brief explanation of the methods of production, properties, and techniques used in applying ultrasonic energy to the eye will be included.

II. THE GENERATION OF ULTRASONIC ENERGY

In medicine, ultrasonic frequencies of 800 and 1,000 kc. are most frequently used. Sound waves of this frequency are most efficiently generated by a quartz crystal or other substances possessing piezo-electric properties.[12-13]

This is accomplished by applying to the crystal an alternating voltage of the same

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Fig. 1 (Baum) Ultrasonic generator used in these experiments (Ultrasonor Model 33). The transducer is resting in the cradle on the left side of the generator.

frequency as the resonant frequency of the crystal, that is, a 1,000-kc. crystal requires a 1,000-kc. voltage. The crystal then converts the electrical energy into mechanical (or sound) energy. A device which converts one form of energy (electromagnetic) to another form of energy (sound or mechanical) is termed a transducer.

The transducer is the source of the ultrasonic energy which is applied to the eye (fig. 1). The transducers used in medicine consist of a metal housing in which the crystal is mounted. They are usually filled with oil which acts as a cooling agent.

III. Properties of ultrasonic energy

Ultrasonic energy is propagated in the form of sound waves whose frequency is greater than 20,000 cps, that is, they consist of regularly spaced rarefactions and compressions. Profound physical, chemical, and biologic alterations can be produced. Physical changes such as cavitation of liquids, heating, dispersion, luminescence, coagulation of aerosols, emulsification, and so forth, may be produced. Hydrolysis, addition, oxidation, polymerization, depolymerization, molecular rearrangement, colloidal dissolution, or peptization, are some of the chemical changes induced by high levels of ultrasonic energy.

Biologic changes such as alteration of membrane permeability, lysis of cells and macromolecules, and modification of the virulence of viruses and bacteria have been reported. Specifically, ultrasonic energy can lyse erythrocytes and macromolecules. Because of this property it has been used in the treatment of vitreous hemorrhages.[14–16]

The coefficient of absorption for ultrasonic energy in gases increases with frequency so that at 1,000 kc., transmission through air is impractical. This physical characteristic of ultrasonic energy necessitates special coupling techniques for radiation of the eye.

IV. Various techniques of coupling ultrasonic energy to the eye

Liquid coupling media such as degassed water, or nonchemically reactive oils or ointments must be used to exclude gas from the system. Because of the multicurved surfaces about the eye, containment of these coupling media is difficult. To solve these problems, the following methods have been tried.[17]

A. Rubber condom technique

A rubber condom filled with degassed water, its outer surfaces coated with petrolatum, is placed over the eye and the transducer applied to it. Movement of the transducer with this arrangement is very simple and constant motion of the transducer prevents local overheating of tissue. Unfortunately, approximately 60 percent of the energy may be lost when using this method of coupling. Clinically, this is the simplest and safest method (fig. 2-A).

B. Via fluid

The lower end of a glass funnel is shaped to conform to the shape of the eye so that a sealed system results. The funnel is then filled with degassed normal saline or 0.25-percent methyl cellulose. The transducer is immersed in the liquid and the energy is transmitted through the fluid to the eye (fig. 2-B). Coupling with this method results in a 90-percent loss of the generated energy.

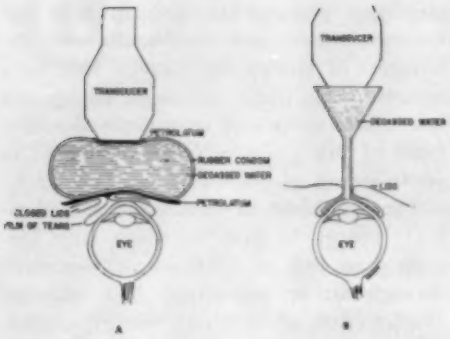

Fig. 2 (Baum). Methods of coupling ultrasonic energy to the eye. (A) Via rubber condom filled with degassed water and coated with petrolatum. (B) Via fluid contained in funnel.

C. Lid application

The transducer may be coated with petrolatum and applied directly to the lids. The film of tears acts as the final coupling layer. Two disadvantages are present. The lids absorb energy and there is as much danger of injuring the lids as there is the eye.

D. Direct application

The radiating surface of the transducer is coated with petrolatum or mineral oil and applied directly to the surface of the anesthetized eye. Such application may result in localized overheating of tissues. This may be overcome by pulsing the ultrasonic energy or constantly moving the transducer.

All methods of coupling other than direct stationary coupling result in marked loss of ultrasonic energy, so that the resulting tissue dose is reduced to "safe" limits.

V. Dosage limits

Previous experiments established that a tissue dose of 0.5 w./cm.^{2}/5 min. at 1,000 kc. direct coupling via petrolatum from the center of the transducer does not produce any permanent ocular damage.[18]

The details of these experiments and the pathologic changes which higher levels of radiation may produce have been reported elsewhere. The present study was conducted within the above limitations in dosage.

VI. Factors affecting the lysis of red blood cells by ultrasonic radiation

The concentration of red blood cells, total volume irradiated, viscosity of suspending medium, frequency of ultrasonic wave, species of red blood cell, resonant points, temperature, concentration of gases in suspending medium, all affect the rate at which lysis occurs.

A. The lysis of suspended red blood cells

Radiation of red blood cell suspensions, diluted 1:1,000 in 50 cc. quantities at 1,000 kc. resulted in some lysis of red blood cells if the temperature was permitted to rise. The degree of lysis was measured on a Rouy Photometer using a 550 mμ filter and examining 5.0 cc. of fluid every five minutes during the course of the exposure. Human erythrocytes lysed more readily than rabbit red blood cells under these conditions.

VII. Experiments with injected autogenous blood

Material. Male, pigmented rabbits approximately four to five lb. in weight were used.

Procedure. Freshly drawn blood was collected with acid citrate dextrose solution as the anticoagulant. Whole blood was used rather than a suspension of erythrocytes because duplication of naturally occurring hemorrhages was desired. Varying quantities of this freshly drawn autogenous blood were injected through the pars plana into a healthy rabbit eye using a 25-gauge needle. The acid citrate dextrose-blood mixture did not produce any pathologic changes other than might be expected from the trauma of the injection.

A. Determination of the rate of spontaneous absorption of blood from the vitreous of the rabbit

Intravitreal hemorrhages frequently absorb spontaneously. The rate at which whole blood absorbs from the vitreous of the rabbit

TABLE 1

RATE OF SPONTANEOUS ABSORPTION OF BLOOD FROM VITREOUS OF RABBIT

Animal No.	Quantity of Blood Injected into Vitreous* (cc.)	Eye	Results
24	0.01	O.D. O.S.	Blood cleared in 141 da. Blood cleared in 21 da.
25	0.05	O.U.	Blood still present 186 da. after injection O.D. > O.S.
21	0.10	O.D. O.S.	Cleared in 30 da. Blood present on the 99th da.

* The blood used in these experiments was autogenous blood consisting of a mixture of 0.85 cc. blood to 0.15 cc. acid citrate dextrose solution (ACD) which contains 26.7 gm. trisodium citrate, (5.5 H_2O), 8.0 gm. citric acid and 22.0 gm. of dextrose/liter. As described in: Cohen, E. J., et al.: A system for separation of the components of the human blood. J. Am. Chem. Soc., **72**:465, 1950.

has, to the best of my knowledge, not been reported. To determine this rate in the rabbit, varying quantities of autogenous blood (table 1) were injected intravitreally. No treatment was administered. No correlation between the amount of blood injected and the time required for its spontaneous absorption was noted. The rate of spontaneous absorption was so slow that it would not interfere with the course of these experiments.

B. Experiments with ultrasonic energy to increase rate of absorption

Characteristics of ultrasonic generator. An Ultrasonor Model No. 33 was used for these experiments. This generator is specifically constructed for medical use. It has the following characteristics: Energy density 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 watts per square cm.; the total sound output equivalent is equal to 1.25, 2.5, 5.0, 7.5., 10.0, 12.5, and 15.0 watts; the frequency is 1,000 kc. (one million cycles per second); the impulse ratio was 1:5, 1:10, and 1:20, that is the generator delivers power for one fifth of a second and is off for four fifths second, for one tenth of a second and off for nine tenths second, and so forth. To obtain a constant output the voltage regulating equipment shown in Figure 3 was required.

Procedure. The same techniques as those described in Section VII-A were used for the collection and injection of the blood into the vitreous. However, this time the right eye served as the control and the left eye was irradiated. The details of the experiments are listed in Table 2.

1. Results obtained with direct coupling

No difference in the rate of absorption between the control and treated eye could be noted in these animals when they were

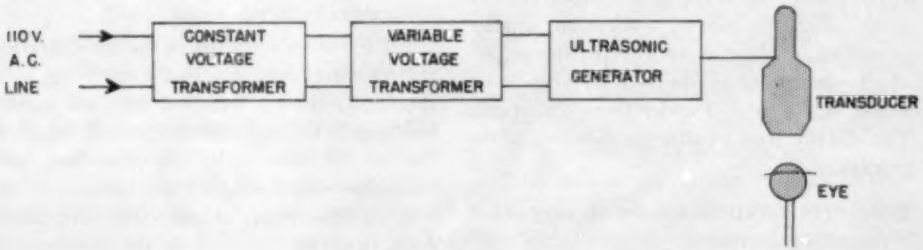

Fig. 3 (Baum). Block diagram of the electronic components required to stabilize the output of the ultrasonic generator.

TABLE 2

EFFECT OF ULTRASONIC RADIATION UPON RATE OF ABSORPTION OF BLOOD FROM VITREOUS OF THE RABBIT

Rabbit No.	Amount of Blood (cc.)	Unit Dose‡ w./cm.² time (min.)	Number of Exposures	Total Radiation Time (Min.)	Days Elapsed Between Injection of Blood and Start of Ultrasonic Radiation	Result	Total Days of Observation
83	0.1 O.U.	0.5*	24	120	3	Amount of clearing seemed equal, O.U.	75
79	0.1 O.U.	1.0*	22	110	5	Treated eye slightly clearer than control	75
39	0.05 O.U.	1.0*	14	70	1	Treated eye slightly clearer than control	48
20	0.01 O.U.	0.5*	1	5	14	Treated eye cleared before control	101
		1.0†	8	24			
				29			
23	0.01 O.U.	0.5*	1	5		Treated eye cleared slightly more rapidly than control	162
		1.0†	9	27			
				32			
32	0.05 O.U.	0.5, O.U.*	4	20	1	Despite equal Rx of both eyes, O.S. cleared before O.D.	62
		1.0, O.U.†	10	30			
				50			

* Unit dose of 0.5 w./cm.²/5 min.
† Unit dose of 1.0 w./cm.²/3 min.
‡ Direct coupling was used in all these experiments. The left eye was irradiated and the right eye served as a control except where indicated.

treated at an intensity of 0.5 w./cm.²/5 min. The intensity of the radiation was therefore increased to 1.0 w./cm.²/3-5 min. despite the danger of ocular injury. At this higher level of irradiation, absorption of the vitreous hemorrhage occurred in the treated eye at a slightly faster rate than in the control, but only after a prolonged waiting period. The higher energy levels did produce permanent corneal scars. Since the concentration of red blood cells in the vitreous theoretically would affect the rate of absorption, smaller quantities of blood were injected intravitreally to determine if the rate of absorption would be accelerated under these conditions. This did not seem to alter the slowness of the process.

2. RESULTS OBTAINED WITH THE ALTERNATE COUPLING METHODS

Five other rabbits were simultaneously irradiated using the other coupling methods already described. No difference in the rate of absorption was noted under these conditions. Coupling via a condom, funnel, or the lids reduces the intensity of the ultrasonic energy reaching the vitreous. This probably accounts for the negative results. Since the results were uniformly negative tabulation of the experiments has been omitted.

3. RESULTS OBTAINED WHEN THE VITREOUS IS LIQUEFIED BY HYALURONIDASE AND DIRECT COUPLING IS USED

All the cases reported in the literature involved chronically diseased eyes. Such eyes frequently have a liquefied vitreous. Pirie[19] and von Sallmann[20] have reported liquefaction of the vitreous by hyaluronidase. Von Sallmann also reported some increase in the rate of absorption of suspended red blood cells from the vitreous in the presence of hyaluronidase.

Method. Hyaluronidase was injected intra-

TABLE 3

EFFECT OF ULTRASONIC RADIATION UPON RATE OF ABSORPTION OF BLOOD FROM RABBIT VITREOUS LIQUEFIED BY HYALURONIDASE

Rabbit* No.	Days Elapsed Between Hyaluronidase Injection and Injection of Blood	Number of Days Between Hyaluronidase Injection and Start of Ultrasonic Irradiation	Unit Dose w./cm.²	Number of Exposures	Total Exposure Time	Difference Between Control and Treated Eye	Total Time of Observation (days)
9	13	21	1.0/3 min.	8	24 min.	None	146
11	14	18	1.0/3–5 min.	15	49 min.	More blood in treated than in control eye	177
12	0	30	1.0/3 min.	8	24 min.	None	59

* 0.05 cc. of blood collected with ACD injected O.U.

Fifty turbidity units of hyaluronidase were injected intravitreally into each eye. Only direct coupling was used in these experiments. For brevity, the reactions to hyaluronidase have been omitted.

Rabbit 9 received 37.0 mg. cortisone subconjunctivally, Rabbits 11 and 12 each received 12.0 mg. cortisone subconjunctivally.

vitreally to liquefy the vitreous of rabbits. Autogenous blood was then injected intravitreally and ultrasonic radiation applied after varying waiting periods. This variable waiting period eliminated the possibility that the combination of hyaluronidase and blood alone would produce rapid clearing of the hemorrhage from the vitreous. The lapse of time between the injection of the hyaluronidase and the injection of blood did not facilitate the absorption of blood. Because permanent eye damage was produced by radiating the eye at 1.0 w./cm.²/5 min., the time was reduced in this group to a maximum of three minutes. Under these conditions the results were consistently negative. Table 3 furnishes the details of these experiments.

VIII. Discussion of the animal phase

Th experimental results were consistently negative, unless the "safe" tissue dose was exceeded. Even under these circumstances only minimal acceleration of the absorption rate resulted. An increased rate of absorption was noted only in those eyes which received such high doses of ultrasonic energy that a marked increase in temperature resulted. Eyes not receiving these high doses showed no response. Cavitation requires such high energy levels at a frequency of 1,000 kc. that it cannot be considered to have played a role in these experiments.

Quantitative evaluation of the rate of absorption by tagging erythrocytes with radioactive iodine or phosphorus proved impractical. Iodine was excreted from the vitreous in 24 hours and the half-life of phosphorus was too short. Hemoglobin measurements were so inconclusive that they too were abandoned and these results are omitted from this report. Since a gross effect, namely a rapid clearing of the injected blood, was sought, qualitative evaluation is adequate.

Because of the difficulty of applying the results obtained in animals to the human, treatment of a patient was undertaken.

IX. Case report

Mr. L. H., a 60-year-old white man, was a patient on the medical service for hypertensive vascular disease probably secondary to renal disease. He also had diabetes mellitus and left hemiparesis following a cerebrovascular accident four and one-half years prior to this admission. The patient states that following this cerebrovascular accident he became totally blind in his right eye.

The pertinent eye findings immediately prior to ultrasonic irradiation were:

O.D., no light perception; O.S., 20/50 correctible to 20/20, J1. The right eye showed a normal cornea and anterior chamber. There was sclerosis of the lens with early posterior subcapsular cataract formation. The vitreous

was fluid and had a haze characteristic of hemorrhage. The anterior vitreous had coarse floaters and large clumps, which, because of their size, were assumed to be blood, but could not positively be identified as such. The posterior vitreous was so dense that the slitlamp beam could not penetrate.

Fundi O.D., no light reflex due to massive vitreous hemorrhage; O.S., normal except for A/V 1:2, widening of the light reflex, third-grade AV nicking. Absence of foveal reflex. No hemorrhages or exudates.

Course of treatment

The patient received one radiation of 1.0 w./cm.^{2}/3 min. applied directly to the closed lids with vaseline as a coupling medium. The patient had no complaints during the irradiation. Immediately following radiation an iritis developed. This persisted despite cortisone therapy. On the 36th day the disc and some blood vessels could be discerned. The disc was pale and the vessels attenuated. Encouraged by this change, further irradiation was administered despite the persistent iritis.

The dose of ultrasonic radiation was now increased to 3.0 w./cm.^{2}/5 min., but the coupling was changed to a rubber condom filled with degassed water and coated with petrolatum. Ten such doses were administered

After the fifth treatment the optic disc and the macula were well visualized. Optic atrophy and two lesions of the macula were observed. Progressive clearing of the vitreous was noted through the seventh dose. No other change was noted other than miosis which may have been due to edema of the iris.

During the treatments the patient experienced only a slight sensation of warmth and tingling, but no pain. The ocular tension dropped following each treatment. This may have been due to the weight of the transducer pressing upon the eye. The iritis persisted but was composed primarily of large cells (suggestive of displaced pigment) with no flare. The cellular element of the iritis did not respond to intensive cortisone therapy. Upon conclusion of this series of treatments, there remained a large vitreous opacity. When this mass floated up, the fundus could be seen behind it.

Forty-two days after the last radiation, $\pm$4.0 cells were still present in the anterior chamber. The density of the vitreous opacities seemed to be increasing so that it was again difficult to view the fundus.

Another course of radiation was advised, but the patient failed to return.

Discussion

The sensations which the patient experienced closely paralleled those described elsewhere in the literature. No glaucoma or cataractous changes were produced during the period of observation. Whether the cells in the anterior chamber represented a true iritis or merely the displacement of pigment is a question which cannot be answered, other than to state that cortisone had little influence upon it.

X. Conclusions

Ultrasonic radiation within the "safe" tissue dose does not influence the rate of absorption of vitreous hemorrhages in the rabbit. Greater intensities of ultrasonic energy produce only a slight acceleration of the rate of absorption. Liquefaction of the vitreous by the injection of hyaluronidase, followed by ultrasonic radiation, did not modify the rate of absorption. The differences between the results obtained in the human and the rabbit may be explained in part by hypothecating that in the human ultrasonic radiation may simply dislodge and fragment the vitreous opacity (as this may be accomplished at low levels of ultrasonic radiation) thus reducing the degree of opacification. Whether other and what other mechanisms may contribute to these differences cannot be definitively answered at this time.

In the human, temporary visualization of the fundus was possible, but total clearing did not occur. Ultrasonic radiation of the

human eye with a massive, persistent vitreal hemorrhage should for the present be attempted only in hopeless cases in which other forms of therapy have failed.

Current studies have demonstrated that the lens absorbs large quantities of ultrasonic energy and that erythrocytes possess resonant modes at which they are especially susceptible to lysis even at low energy levels. By rotating the lens out of the path of a focused ultrasonic beam of the correct frequency increased lysis of erythrocytes might be produced without damaging the eye. These methods of application are to be investigated and may yield more positive results.

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Discussion

Dr. Ludwig von Sallmann (Bethesda, Maryland): Dr. Baum has extended his studies of the use of ultrasound in ophthalmology to an area which remains to be linked to one of the most pressing therapeutic problems of eye disease. No doubt any experimental search for means that promise to speed the resorption of blood or other pathologic deposits from the vitreous deserves credit, particularly when such search is carried out with a critical attitude and the results interpreted with caution, as in the study by Dr. Baum.

My contact with investigations of this kind is limited to a few unpublished experiments of Anthony Donn, who aimed to disrupt hemorrhagic membranes and dense strands in the rabbit's vitreous with a focused ultrasound beam. He failed to obtain positive results, and in addition demonstrated widespread damage to the retina after a reflecting medium has been introduced into the vitreous chamber prior to the irradiation.

I do not know whether Dr. Baum's proposed technique will circumvent such retinal injury, nor can it be predicted whether hemolysis of red cells or liquefaction of vitreous can be accelerated in vivo with a safe technique. The observations of other workers in this field make the outlook dim as far as the human eye is concerned.

I am sure Dr. Baum is aware of the report of

Funder, which indicates that the results of ultrasound treatment on seven patients were questionable at best, and that the damage to surface structures, particularly degenerative changes of superficial vessels as well as relapses of vitreous hemorrhages, could not be avoided.

Several years ago Dr. Gruen of the same eye department told me that these signs of injurious side effects, the recurrence of vitreous hemorrhages during and possibly due to the treatment, and the unimpressive improvement of the condition in a few patients, were the reasons for discontinuing further ultrasound therapy for vitreous hemorrhages.

A change of technique might provide more favorable conditions, but a plea should be made for serial sectioning, of experimental eyes to exclude retinal and vascular damage, especially when use of a focused ultrasonic beam is contemplated.

Finally, Dr. Baum indicated in this paper the difference of the vulnerability between the normal animal eye and the human eye with a diseased vasculature. This must be kept in mind in selecting a so-called safe dose level. I think this is a point that should perhaps be stressed in Dr. Baum's paper.

DR. GILBERT BAUM (closing): I wish to thank Dr. von Sallmann for his comments.

Because of limited time in which I could deliver this paper I have had to eliminate the caution note contained in the text.

The work of Donn and others has been discussed in previous papers. Donn used such high intensities of focused ultrasonic irradiation that damage was unavoidable under this set of conditions. The low energy levels used in the experiments I reported did not produce any damage in the posterior segment of the eye and at very low intensities no ocular damage was produced.

Human erythrocytes possess resonant modes at which lysis can be produced at low energy levels. The lens absorbs large amounts of ultrasonic energy. By operating at the resonant mode of the erythrocyte and rotating the lens out of the path of the ultrasonic beam, we may be able to produce lysis of intravitreal erythrocytes by such low energy levels that no permanent damage is produced.

OCULAR EFFECTS OF SYSTEMIC SIDEROSIS*

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INTRODUCTION

The term "siderosis bulbi," as introduced by Bunge[1] in 1890, refers to ocular changes incident to the prolonged retention of iron-containing foreign bodies in the eye. There are, to the best of our knowledge, no reports available of ocular effects associated with systemic siderosis.

Systemic siderosis may be defined as the sequelae of abnormally high concentrations of iron in blood and tissues. This can be induced by systemic administration of iron compounds,[2] ingestion of large amounts of dietary iron (as observed in Bantu natives,[3-6] or by a general overload of the body with iron liberated from hemoglobin in cases of multiple blood transfusions [7-9] (systemic hemosiderosis). The pathologic changes in such cases may resemble those of idiopathic hemochromatosis. They have been observed exclusively in human beings and may be associated with liver cirrhosis and rarely diabetes.

Many unsuccessful attempts have been made to reproduce hemochromatosis in animals. The experimental work in this field has been reviewed recently by one of us[10] and an extensive study of iron-overload in dogs has been performed. This study was designed to avoid the objections to previous studies by using larger doses of iron and by prolonging the period of observation for as long as seven years. No changes were found which resembled hemochromatosis. During

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† Fellow in the medical sciences, National Research Council.

TABLE 1

EARLY EFFECTS OF INTRAVENOUS ADMINISTRATION OF SACCHARATED IRON OXIDE IN DOG EYES

Animal Number	Single Dose (mg./kg.)	Total Dose (gm./kg.)	Number of Injections	Period of Treatment (days)	Time of Death	Degree of Gomori's Iron-Stain in					Content of Fe in eye† (mg./gm. wet weight
						Lens	Uvea	Pigment Epithelium	Retina	Blood	
7	40	0.04	1	—	1 hr.	−	+	(±)	−	++ (retina)	0.0154
8	40	0.04	1	—	4 hr.	−	±	(±)	−	+	0.0067
9	40	0.04	1	—	8 hr.	−	(±)	(±)	−	±	0.0081
10	40	0.04	1	—	1 da.	−	(±)	−	−	−	0.0059
11	40	0.04	1	—	4 da.	−	(±) Granular	−	−	−	0.0055
12	40	0.04	1	—	24 da.	−				−	0.0053
13	20	0.72	36	72	4 mo.	−	++	±	−	−	0.0197
1*	13–18	2.5	143	195	7 mo.	−	++	+	−	−	
15	20	0.8	40	80	Alive						
16	20	0.84	42	84	Alive						
17	20	0.84	42	84	Alive						
18	20	0.84	42	84	Alive						

* Identical with dog 1 in Table 2.
† Normal Fe content in dog eye 0.01 (Av.) as compared to 0.02 mg./gm. wet weight in brain.

this study, however, it was observed that dogs kept for a period of about four years after systemic administration of iron exhibited signs of impaired vision. This observation initiated the present investigation of the ophthalmologic aspects of experimentally induced systemic siderosis.

MATERIAL AND METHODS

Healthy, full-grown mongrel dogs from different litters were subjected to repeated intravenous injections of saccharated iron oxide* or multiple transfusions of compatible blood. The weight of the animals varied from 5.9 to 17.5 kg. The total doses of iron administered over a period as long as 300 days ranged from about 13 mg. to 2.5 gm./kg. body weight. Pertinent data referring to the individual amounts are given in Tables 1, 2, and 3.

The examinations of the fundi were performed with direct and indirect ophthalmoscopy. The animals were killed and the eyes

* The commercial preparations used were Ferrivenin-Benger's Ltd. lot No. 10369.

TABLE 2

DELAYED EFFECTS OF PROLONGED INTRAVENOUS ADMINISTRATION OF IRON* IN DOG EYES

Animal Number	Single Dose (mg./kg.)	Total Dose (gm./kg.)	Number of Injections	Period of Treatment (days)	Time of Death	Degree of Histopathologic Change				Content of Iron	
						Lens	Uvea	Pigment Epithelium	Retina	Fe-Stain	mg./gm.
1	13.0–18.0	2.5	143	195	7 mo.	−	−	−	−	++	
2	9.0–27.0	0.51	37	55	6 yr.	+	±	+++	+++	+	0.18
3	14.0–34.0	1.04	43	66	6¾ yr.	−	±	+++	+++	+	0.27
4	13.0–27.0	0.91	27	44	7 yr.	−	±	+++	+++	+	0.02
5	8.9	0.47	53	180	6 yr.	−	+	++	++	±	0.02
6	6.4	0.47	73	315	7 yr.	+	±	+++	+++	±−+	0.02

* Animals #1–4 received commercial preparation of saccharated iron oxide ("Ferrivenin"—Benger's Ltd. let 10369, "Inferon"); animals 5 and 6 transfusions of matched blood.

TABLE 3

STAINABILITY FOR IRON IN THE CILIARY PROCESS OF RABBITS AFTER A SINGLE INTRAVENOUS INJECTION OF 200 MG. OF SACCHARATED IRON OXIDE

Number of Rabbit	Time of Death	Diffuse Stromal Stain for Iron	Granular Iron Deposits in Histiocytes or Endothelium
1	1 hr.	++	−
2	2 hr.	+	±
3	4 hr.	±	+
4	8 hr.	(±)	+
5	24 hr.	−	++
6	2 da.	−	++
7	4 da.	−	++
8	7 da.	−	+
9	10 da.	−	+
10	14 da.	−	++
Total 10	1 hr. to 14 da.	(±) to ++	± to ++

enucleated immediately following death. The right eyes were fixed in 10-percent formalin or Zenker's solution. The histologic sections were bleached and stained with hematoxylin-eosin and Gomori's Fe-staining technique.[11] As counterstain in the latter technique, a one-percent aqueous solution of nuclear fast red (Masson's Biebrich Scarlet)[12] was employed. The left eyes were thoroughly cleaned of adherent tissue and prepared for chemical analysis of their iron content. Diluted samples of entire eyes were analyzed colorimetrically using a potassium-thiocyanate—method as described by Moore et al.[13]

The eyes of five normal mongrel dogs of equivalent age were used as controls in the study of the histopathologic changes.

In another series of experiments, 16 albino rabbits were subjected to a single intravenous injection; six other albino rabbits were given repeated injections of 200-mg. saccharated iron oxide.* The animals were killed as indicated in Tables 3 and 4. Their eyes were prepared in the same manner as those of the dogs.

Histologic sections of two human eyes, obtained in autopsies of patients treated with repeated blood transfusions for severe anemia, were stained with Gomori's technique and examined for ocular signs of systemic hemosiderosis.

RESULTS

I. OPHTHALMOSCOPIC OBSERVATIONS

Ophthalmoscopic examination of the animals subjected to intravenous injections of saccharated iron oxide (table 1, dogs 7 to 18) revealed no changes of significance during the period of injections and thereafter for seven months. At about this time, attenuation of the blood vessels appeared in one of the seven dogs subjected to repeated iron injections.

Optic atrophy and degenerative changes in the retina were present in all dogs observed for five years or more (table 2). The ophthalmoscopic appearance in these cases resembled that of siderosis bulbi and retinitis pigmentosa. Similar changes have appeared in the two dogs which had received repeated transfusions of blood (5 and 6 in table 2). In addition, four of the six long-term dogs exhibited an asteroid hyalitis. Two dogs (2 and 3) also showed cataractous changes consisting of vacuoles of small or medium size located particularly in the post equatorial area. Siderotic discoloration of the refractive media or uveal tissues was not recognized with the hand slitlamp or ophthalmoscope. Unfortunately, due to the fact that the initial observations were made on dogs six years after iron administration, the progression of changes between one and six years remain to be defined. Animals 15 through 18 (table 2) will be kept under further observation to fill this gap.

II. HISTOLOGIC CHANGES

Before describing the histologic changes observed in the eyes of the dogs exposed to systemic siderosis or hemosiderosis, a review of some histo-anatomic features of the normal dog eye might be relevant. Figure 1 is a photomicrograph of the retina of a normal dog. Its neural structure is similar to that of a human retina. The retinal pigment epi-

* Dr. Tsuyoshi Yamashita assisted in the performance of these experiments.

TABLE 4

DISTRIBUTION OF SIDEROSIS IN OCULAR STRUCTURES OF RABBITS AFTER SINGLE OR REPEATED INTRAVENOUS INJECTIONS OF 200 MG. OF SACCHARATED IRON OXIDE*

Number of Rabbits	Total Dose of Iron (mg.)	Time of Death	Plasma Siderosis	Cytosiderosis†		
				Iris	Ciliary Processes	Choroid
1	250	2 da.	−	+	++	(±)
1	400	2 da.	++	+	+++	±−+
1	600	3 da.	++	+	++++	+
1	800	4 da.	++	+	++++	+
1	1000	5 da.	++	+	++++	++
1	1200	6 da.	++	+	++++	++
2	200	3 wk.	−	−	(±)	−
2	200	4 wk.	−	−	(±)	−
2	200	6 wk.	−	−	(±)	−
12	200 to 1200	2 da. to 6 wk.	− to ++	− to +	(±) to ++++	− to ++

* The repeated injections have been given in doses of 200 mg. a day. The animals were killed six to eight hours after the last injection.
† Retina, optic nerve, and cornea were free of iron deposits.

thelium and choroid, however, are distinguished by an architecture which differs quite characteristically from that of human eyes. The choriocapillaris is relatively poorly developed and closely attached to the base of the pigment epithelium. The lumen of the choriocapillaris is so fine that passage of more than one erythrocyte at a time seems to be unlikely. The choriocapillary network is fed by slightly larger vessels which originate from major choroidal vessels which pass perpendicularly through the lamellae of the tapetum lucidum. There, they form pillarlike structures which are surrounded by connective tissue (fig. 2). The schematic drawing of Figure 3 may help to illustrate the anatomic situation as it appears in the dog eye in the area of tapetum lucidum. The vessels in these pillarlike structures appear to be particularly prone to obliteration due to swelling of the endothelial cells engorged with phagocytized hemosiderin.

III. PATHOLOGIC CHANGES FOLLOWING SINGLE INJECTIONS OF SACCHARATED IRON OXIDE

Six dogs (table 1) and 10 rabbits (table 3) were subjected to a single intravenous injection of saccharated iron oxide. One hour after the injection, sections stained with Gomori's technique exhibited a diffuse bluish color of the plasma (plasma siderosis) (figs. 4 and 5). The intensity of this blue stain of the plasma decreased with time and was hardly noticeable in the eight-hour sections. At that time, however, the ground substance of the choroid and ciliary processes displayed a diffuse stainability for iron

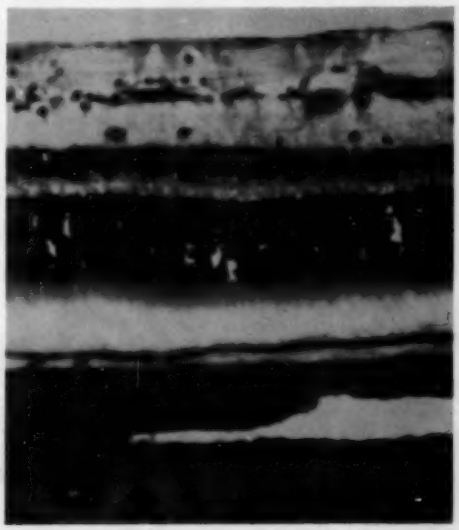

Fig. 1 (Cibis, Brown and Hong). Retina and choroid of normal dog. (Formalin [10 percent], hematoxylin and eosin, ×250.)

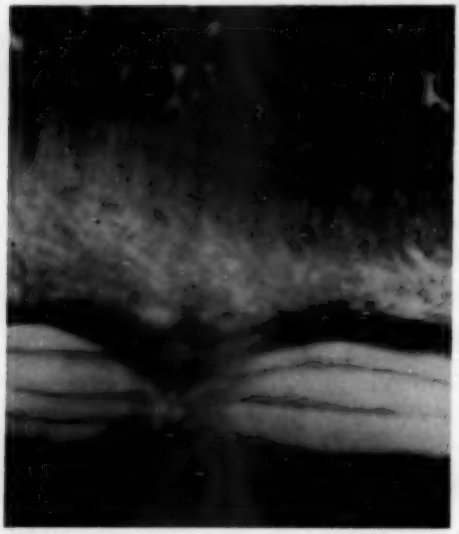

Fig. 2 (Cibis, Brown, and Hong.) Histologic section of cellular tapetum lucidum, retinal pigment epithelium, and outer layers of retina of a normal dog. In the center, pillarlike connection between choroid and choriocapillaris traversing at an angle of about 15 degrees to the perpendicular radius forming the so-called stellulae of Winslow. (Hematoxylin and eosin, ×600.)

which persisted for about 24 hours. Very small amounts of iron were also detected in the pigment epithelial cells of the ciliary processes and the retina at this time (cytosiderosis).

In the sections made on the fourth day after the injeection or later, the diffuse stromal stain had almost vanished. Only granular iron deposits remained (fig. 6). In the ciliary processes they were located in the endothelial cells of the capillaries and stromal histiocytes. The amount of granular iron deposits slowly decreased with time. However, even after months iron-stained granules were still present. No siderin was noted in the neural structures. This seems to be of some significance since retinal degeneration represents a common feature of the late changes in systemic siderosis produced by repeated intravenous injections of saccharated iron oxide. Inflammatory reactions to iron were not apparent at any time.

IV. Pathologic changes following repeated injections of saccharated iron oxide

The histopathologic changes observed in the cases of repeated intravenous injections of saccharated iron oxide occurred in three more or less distinct phases:

1. A siderotic-nondegenerative or early phase, where stainable iron is present but without evident damage to tissues.

2. A siderotic-degenerative or intermediate phase, where stainable iron and tissue damage are present.

3. A nonsiderotic-degenerative or late phase, where tissue damage has occurred but no stainable iron is present.

The transformation from one phase to the next overlapped and therefore the above classification has limited usefulness. A simi-

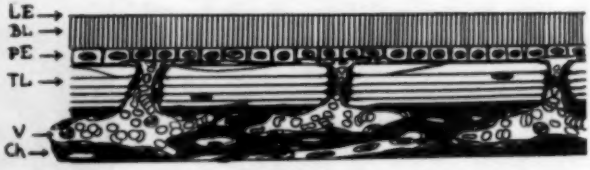

Fig. 3 (Cibis, Brown, and Hong). Schematic drawing of anatomic and histologic relationship between choroid and choriocapillaris at site of the cellular tapetum lucidum. (LE) External limiting membrane (BL) Bacillary layer. (PE) Pigment epithelium. (TL) Tapetum lucidum cellulare with lamellar structures of the cell walls penetrated by pillars of connective tissue with capillaries connecting choroidal vessels with the choriocapillaris. (V) Vessels in choroid. (Ch) Choroidal tissue with heavily pigmented chromatophores.

lar classification can also be applied to the pathologic changes which are associated with or follow other forms of siderosis or hemosiderosis bulbi.

The siderotic-nondegenerative ocular changes in the early phase of systemic siderosis following repeated intravenous injections of saccharated iron oxide resemble, from a qualitative viewpoint, those observed in cases of single injections. From a quantitative viewpoint, however, there were significant differences noticeable. In animals with repeated injections, the stroma of the uveal tissue stained more intensely and the granular iron deposits in the endothelial cells and stromal histiocytes were present in greater amounts even several months after the last injecion (1 and 13). The terminal branches of the short and long posterior ciliary arteries were a preferential site of the granular iron deposits. In many places the swelling of the iron-laden endothelial cells in the choriocapillaris was so great that passage

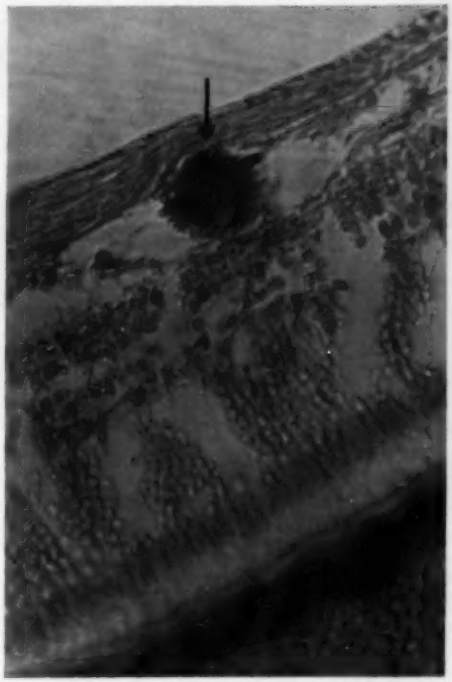

Fig. 5 (Cibis, Brown, and Hong). Retina of dog one hour after intravenous injection of saccharated iron oxide (40 mg./kg.). Gomori's iron stain. Note intensity of diffusely stained plasma protein in retinal vessel indicated by arrow. (Prussian blue reaction, ×330.)

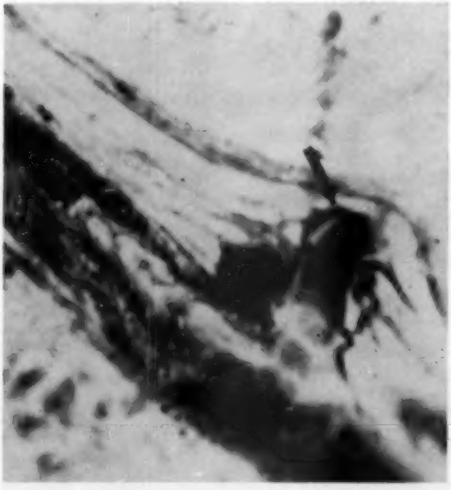

Fig. 4 (Cibis, Brown, and Hong). Choroid and tapetum lucidum cellulare of a dog, four hours after intravenous injection of saccharated iron oxide (40 mg./kg.). Gomori's iron stain. A clot of diffusely iron stained plasma protein fills out the capillary vessel traversing the tapetum as indicated by the arrow. (Dog 8, ×600.)

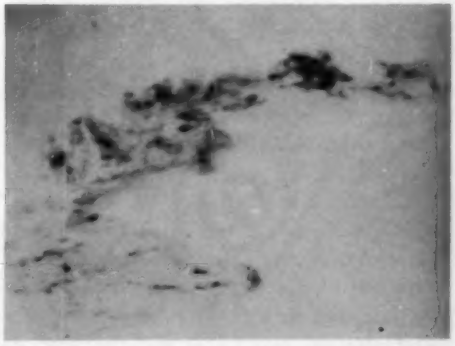

Fig. 6. (Cibis, Brown, and Hong). Granular iron deposits accumulated in endothelial cells of the capillaris and stromal histiocytes of the ciliary processes, seven days after a single intravenous injection of saccharated iron oxide (40 mg./kg.). (Albino rabbit. Gomori's iron stain, ×330.)

Fig. 7 (Cibis, Brown, and Hong). Posterior aspect of the anterior segment (at left) and anterior aspect of the posterior segment (at right) of the eye of an albino rabbit killed about eight hours after the last injection of 200 mg. iron given daily over five days. Gomori's stain for iron. Ciliary processes heavily stained, uvea moderately stained, and retina and iris free of stain except the retinal vessels. Lens has been removed. Note artificial detachment of retina in the upper half at left segment.

of blood cells appeared impossible. Particularly noteworthy is the fact that some of the blood vessels passing through the pillar-like structures in the tapetum lucidum appeared completely obstructed.

Fine granular deposits were also found in the pigment epithelial cells of the retina and the ciliary processes. Iron granules have also appeared in considerable numbers within the cytoplasm of the chromatophores of the choroid and the ciliary processes (fig. 7). With the exception of the cytoplasmic swelling in some of the ganglion cells (fig. 8), the neural elements of the retina appeared normal for as long as seven months. At this time, however, such degenerative changes as cytoplasmic swelling, beginning pallor of the pigment granules, and occasional proliferation of the pigment epithelial cells began in the pigment epithelium. The pathology at this stage had entered the phase of siderotic-degenerative changes.

Due to the circumstances under which this study had been started, we are, at the present time, not in a position to present data concerning the various steps in the development of the degenerative changes in retina, pigment epithelium, and choroid which lead to the appearance of the late stage, five to seven years after conclusion of the injections (table 2).

V. Late pathologic effects of prolonged systemic siderosis

The histologic examination of four dogs which had been killed six to seven years after intravenous administration of saccharated iron oxide, and of two dogs subjected to multiple blood transfusions (table 2), revealed most interesting pathologic

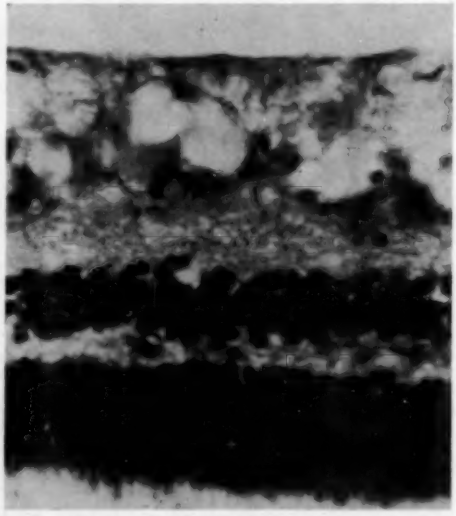

Fig. 8 (Cibis, Brown, and Hong). Retina of dog, seven months after repeated intravenous injections (143) of saccharated iron oxide (2.5 gm./kg.). Cytoplasmic swelling of ganglion cells. (Dog 1 of Table 1. Hematoxylin and eosin, ×440.)

changes. The retina was found to be completely degenerated in all cases. Figure 9 represents a photomicrograph of the posterior segment showing atrophy of the optic nerve and gliosis of the adjacent retina. Figure 10 demonstrates the loss of the neural elements and advanced gliosis with migration of pigment cells into the retina.

The pigment epithelium itself displayed signs of dystrophy in all advanced cases. At many places, it vanished completely or was transformed into migratory cells which surrounded the blood vessels in the retina. The degenerated retina, in general, appeared fused with the underlying choroid wherever the pigment epithelium had disappeared. Proliferation and hypertrophy of degenerated pigment epithelial cells were found in some sections (fig. 11); drusenlike deposits of eosinophilic staining material were seen in others (fig. 12). The pigment in the remaining epithelial cells appeared paler as more degeneration and atrophy of the retina had progressed.

Relatively small amounts of granular iron deposits were found at the late stage of retinopathy in the sections stained with Gomori's Fe-stain (fig. 13). Only in a few cases were clumpy iron deposits detected in endothelial cells of the choriocapillaris and in tributary vessels passing through the

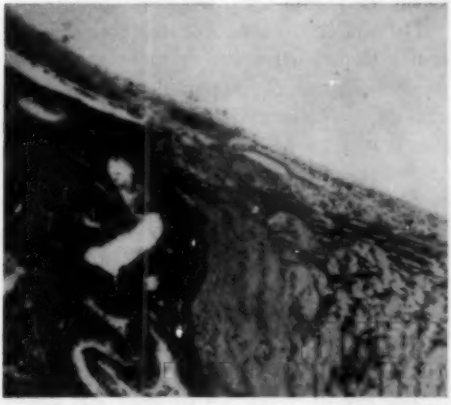

Fig. 9 (Cibis, Brown, and Hong). Atrophy of optic nerve and degeneration of the tapetal retina of a dog, seven years after repeated intravenous injections of saccharated iron oxide (1.04 gm./kg.). (Dog 4. Hematoxylin and eosin, ×60.)

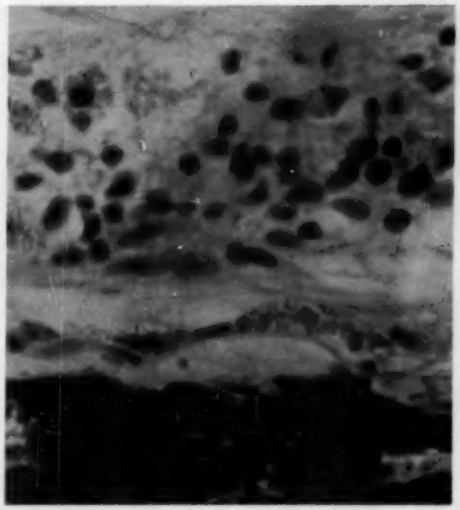

Fig. 10 (Cibis, Brown, and Hong). The same eye as Figure 9. Complete degeneration of the retina, advanced gliosis, and degeneration of the pigment epithelium. (Hematoxylin and eosin, ×600.)

Fig. 11 (Cibis, Brown, and Hong). Complete degeneration of the retina of a dog about seven years after repeated intravenous injections of saccharated iron oxide. Note proliferation and hypertrophy of retinal pigment epithelium in area indicated by arrow. (Dog 4. Hematoxylin and eosin, ×60.)

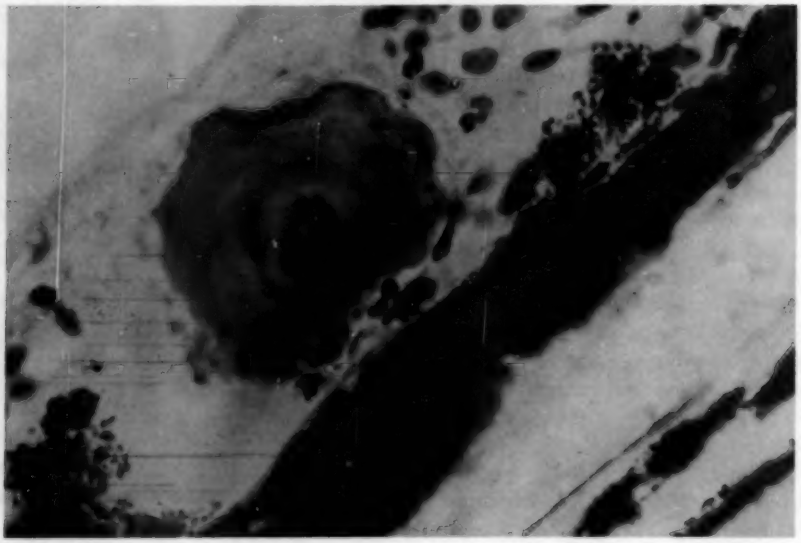

Fig. 12 (Cibis, Brown, and Hong). Drusenlike deposit formed around degenerated retinal pigment epithelial cell displaced in the completely degenerated retina. Degeneration of the pigment epithelium; atrophy of the choroid. (Dog 4. Hematoxylin and eosin, ×600.)

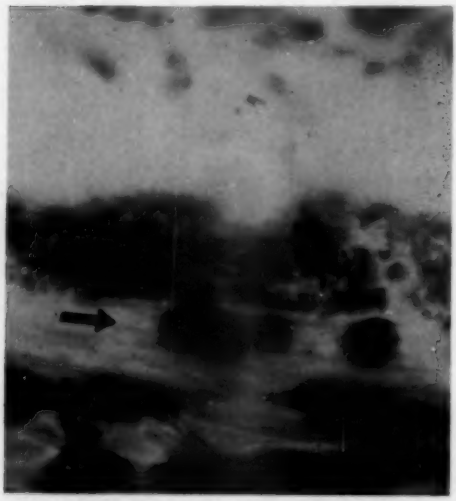

Fig. 13 (Cibis, Brown, and Hong). Black and white photomicrograph of an eye section stained with Gomori's iron stain about seven years after repeated intravenous injections of saccharated iron oxide. Accumulation of granular iron within macrophages or endothelial cells of the choriocapillaris indicated by arrow (Dox 6, ×800.)

pillarlike structures in the tapetum lucidum. Similar deposits were also found in pigment epithelial cells of retina and ciliary body, and to a lesser degree in stromal histiocytes of ciliary processes and endothelial cells of Schlemm's canal. The trabecula sclerae appeared degenerated. No Fe-stain was detectible in the retina, lens, iris, and cornea.

The amount of stainable iron found in the ocular tissues after six or more years was small and not at all comparable to the marked iron deposits observed in dogs killed within the first year after initiation of the prolonged systemic administration of saccharated iron oxide. However, the chemical analysis revealed significantly elevated iron concentrations in two cases (2 and 3) in which the iron values exceeded the average for a normal dog by factors of 18 and 27, respectively.

A chemical analysis of various structures in one eye of a dog (4 of table 2), whose other eye showed retinal degeneration, is given in Table 5. The greatest amount of iron was found in the anterior segment, a

TABLE 5

IRON CONCENTRATION OF OCULAR STRUCTURES OF DOG 4 IN TABLE 2

Tissue	Wet Weight (gm.)	Iron Concentration (mg./gm.)
Lens	0.25	0.00984
Vitreous	1.8	0.00193
Anterior segment	1.27	0.02030
Posterior segment	1.28	0.00635
Total	4.60	0.00836

finding which is in good agreement with the histologic observation of iron accumulation preferentially in the ciliary body.

VI. LATE PATHOLOGIC EFFECTS OF PROLONGED SYSTEMIC HEMOSIDEROSIS

As has been mentioned previously, repeated transfusions of compatible blood produced the same changes in two dogs (5 and 6, table 2) as repeated intravenous injections of saccharated iron oxide (figs. 14 and 15). This observation prompted us to search for ocular defects in human patients subjected to repeated blood transfusions prior to death. Figures 16 and 17 represent photomicrographs of an eye obtained at autopsy from a patient who had received about 150 blood transfusions during treatment for severe aplastic anemia. Complete obstruction of capillaries in the retina and the choriocapillaris could be detected in almost every section stained for iron.

The main site of iron deposits in a granular form was in the endothelial cells or macrophages which appeared swollen and distorted to spheric or hemispheric formations (fig. 18). Another site of preference for the accumulation of iron was the perivascular tissue in the choroid. Of great interest is the fact that a considerable amount of granular iron in smaller particles could be seen in bleached sections within the epithelial cells of the ciliary processes and pars plana (fig. 19). Only very small amounts of iron were seen within the pigment epithelium of the retina. The neural elements of the retina were not affected at the stage of systemic hemosiderosis at which the patient succumbed.

In the absence of evidence for degenerative changes, this eye could be classified as siderotic-nondegenerative. Further evidence for the potentially deleterious effects of iron liberated from the blood either in cases of systemic hemosiderosis or intraocular hemorrhages will be the subject of a later publication.

DISCUSSION

The essential observation here reported is

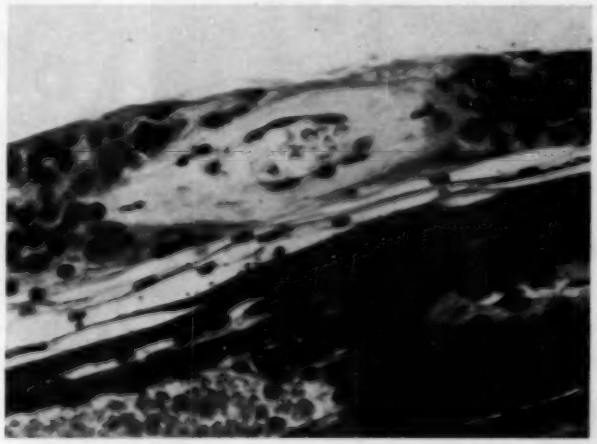

Fig. 14 (Cibis, Brown, and Hong). Degenerated retina of dog 6, seven years after multiple blood transfusions equivalent to a total body iron concentration of 0.47 gm./kg. body weight. In the center of the retina is an oblique section of a hyalinized blood vessel. Degeneration of the retinal pigment epithelium. Pigment containing cells in retina and around the blood vessel. (Hematoxylin and eosin, ×330.)

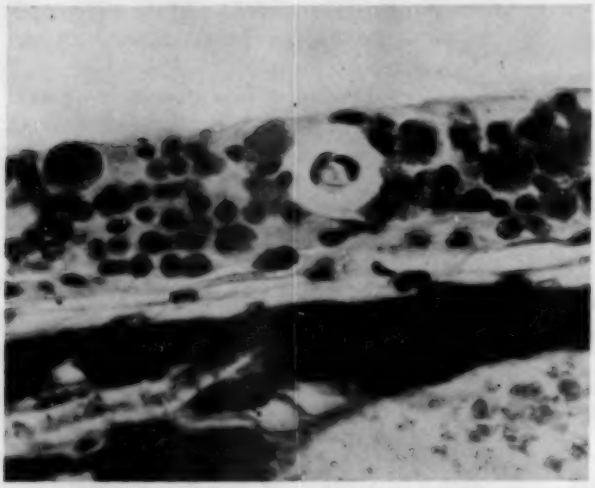

Fig. 15 (Cibis, Brown, and Hong). The same as Figure 14. Hyalinization of a small vessel in the periphery of the retina. Complete degeneration of the retinal pigment epithelium. Phagocytes with pigment and retinal pigment epithelial cells in the retina. Tapetum lucidum in direct contact with the retina. (Hematoxylin and eosin, ×330.)

that systemic siderosis as well as hemosiderosis, induced either by repeated intravenous injections of saccharated iron oxide or by multiple blood transfusions, resulted in degenerative ocular conditions which closely resemble those seen in siderosis bulbi and retinitis pigmentosa. All three of those conditions have in common slow and insidious degenerative changes in retina, pigment epitheilium, and choroid. Not infrequently they terminate in blindness.

It is of prime interest that in cases of siderosis bulbi caused by an intraocular foreign body containing iron, the dissemination of

Fig. 16 (Cibis, Brown, and Hong). Retina of patient with severe aplastic anemia treated with 150 blood transfusions. Gomori's iron stain showing iron deposits blocking the lumen of capillaries in the retina and choriocapillaris as indicated by arrows (×150).

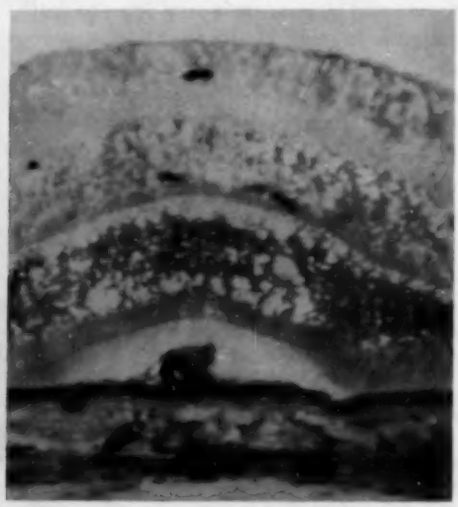

Fig. 17 (Cibis, Brown, and Hong). Same patient as in Figure 16. Gomori's stain of bleached section showing multiple thrombuslike accumulation of granular iron in capillaries of the retina, the choriocapillaris, and chromatophores of the choroid (×300).

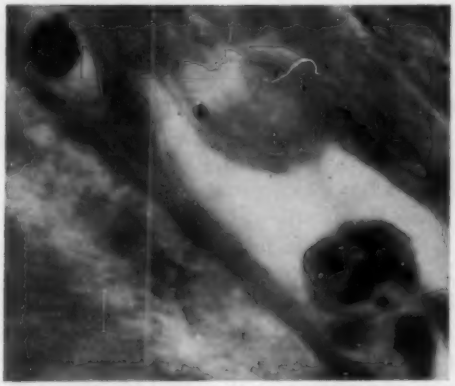

Fig. 18 (Cibis, Brown, and Hong). Same patient as in Figure 16. Macrophages and endothelial cells in choriocapillaris showing big clumps of iron in the cytoplasma. (Gomori's iron stain, ×880.)

dissolved iron in the ocular tissues precedes the degenerative changes by a considerable period of time, sometimes amounting to many years. The same seems to be true in cases of ocular pathology produced by systemic siderosis or hemosiderosis. Consequently, the term "siderosis bulbi" does not necessarily imply that functional and pathologic disturbances must be found in obligatory association with iron depositions in the eye.

For that reason, an attempt has been made to distinguish between (1) a siderotic-nondegenerative phase, that is, a condition of siderosis without functional or histologic manifestations of ophthalmopathy; (2) a siderotic-degenerative phase, that is, a condition of visible siderosis associated with signs of ophthalmopathy presenting themselves in the form of retinal, choroidal, iridal, or lenticular changes (retinopathy, choroidopathy, iridopathy, phakopathy); (3) a nonsiderotic-degenerative phase or siderogenous ophthalmopathy without signs of siderosis in the ocular tissues. Many eyes fall into borderline conditions or overlapping classifications, however.

The question may be raised whether the amount of iron administered in our experiments is comparable to the amount of iron reported in the literature in cases of intraocular foreign bodies and siderosis. Siderotic ocular changes have been reported by Huettemann,[14] Schiøtz,[15] Rychener,[16] and others in cases in which the weight of the intraocular steel splinters found amounted to 0.18 and 0.45 mg., respectively. Assuming an average weight of the eye of 7.0 gm., the potential amount of iron would have been in Huettemann's and Schiøtz's cases 0.025 and 0.065

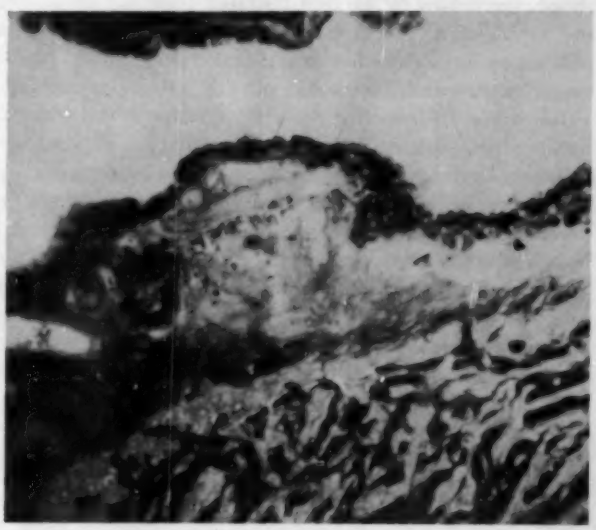

Fig. 19 (Cibis, Brown, and Hong). Same patient as in Figure 16. Ciliary processes with a great number of deeply stained granules of iron within the epithelial cells and, to a lesser degree, stromal histiocytes (Gomori's iron stain, ×150.)

mg./gm. ocular tissue. Such a computation would imply that all iron would have been available in a soluble form and evenly distributed.

As summarized in Table 2, the amount of soluble iron administered intravenously in our long-term experiments ranged from 0.47 to 2.5 mg./gm. body weight. Furthermore, considerably higher concentrations than the average figures given may have been reached in certain tissues with special affinity for iron.

At the time of death, two out of five of the long-term animals revealed values of iron in the ocular tissues which measured 18 to 27 times the normal values, whereas the remaining animals exceeded the average figure by only a factor of two. The iron values in the eyes in three of our long-term dogs can be considered significantly higher than normal. The fact that the iron stain in the histologic sections of the same dogs does not correlate with the high values obtained by chemical analysis remains unexplained.

The implications of the observations made so far are of prime importance from a clinical as well as theoretic viewpoint. It is our belief that many pathologic conditions associated with or following intraocular hemorrhages are caused by the iron liberated from the hemoglobin. A current study of cases afflicted by intraocular hemorrhages and pertinent experimental work has already provided ample evidence for some relationship between hemosiderosis bulbi and secondary glaucoma and retinal degeneration.

Of great interest for the evaluation of the presented data is the observation of blindness in dogs after distemper (Formston[19]). According to Parry,[20] it is probable that most cases of blindness associated with such infections are due primarily to the involvement of the visual system central to the retina. The same author, however, presented unequivocal evidence that some cases were due to retinopathies associated with degeneration of the retina. Of the four main types of primary distemper retinopathy reported by Parry,[21] only one can be considered as similar to ours. It is this type which is distinguished by chronic generalized primary retinopathy with gradual loss of day and night vision during a period of one to two years after the primary infection. Of the dogs used in our experiments, however, only dog 17 had distemper.

Hereditary conditions with similar pathologic ocular manifestations were described by Parry[20] in Irish setters. However, the same author[22] reported in a subsequent paper three cases of generalized progressive atrophy of uncertain etiology in Irish setters in which there was no evidence of heredity, or of distemper-complex virus infections, or glaucoma. The cases were of two types, first, a familial type with two of three litter mates involved, and, secondly, an idiopathic type with no pertinent medical history. In the first type, the dogs were blind when they were 18 months old. The histology of these cases resembled those of hereditary atrophy as observed by Parry in the red Irish setters. The idiopathic type has been described by Parry as unsymmetric and of least severity over the peripapillary tapetal fundus. Cataractous changes in the lens were present in the late phases of both types.

In a later publication, Parry[23] described a central progressive atrophy of the retina with pigment epithelial dystrophy. In his series of 15 cases the dogs developed a central scotoma by early or middle adult life but retained normal peripheral photopic and scotopic vision for years. The principal features of the central atrophy were explained on the hypothesis of an "inadequate blood supply through the choroidal circulation from the short posterior ciliary arteries, followed by slow failure of the supply to the anterior parts of the choroid from the anterior ciliary arteries." Such a hypothesis could well be applied to our cases, whereby the iron could be the potential factor responsible for the disturbance of circulation in the ciliary system.

In this connection it may be pertinent to

mention that Racker and Krimsky[24] observed that iron salts like $FeSO_4$ inhibit glycolysis in the brain. They also observed that the Theiler FA strain of mouse encephalomyelitis virus produced inhibition of glycolysis in direct proportion to the iron content of the virus. The observation as well as others regarding the production of retinopathy similar to retinitis pigmentosa and siderosis bulbi by agents like Iodoacetic acid (Noell[25, 26]) and ionizing radiation[27–30] might indicate that related mechanisms are affected in these cases of unrelated etiology.

Summary

Repeated intravenous injections of saccharated iron oxide or multiple blood transfusions in dogs can produce degenerative ocular conditions closely resembling those seen in man with siderosis bulbi and retinitis pigmentosa.

Siderotic deposits are demonstrated in the retina, retinal pigment epithelium, choriocapillaris, and ciliary body of a patient treated with multiple blood transfusions for aplastic anemia.

A classification for the experimental and clinical observations is presented which correlates siderosis and hemosiderosis with the degenerative changes observed histopathologically.

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Discussion

Dr. David G. Cogan (Boston): I found this paper on siderosis bulbi of great interest. Most of it was new to me. Stimulated by the authors' experiments, I reviewed the slides listed in my files as siderosis bulbi, and I can confirm the authors' observations on the early changes in siderosis. The iron granules are first aggregated conspicuously in the endothelial walls of the blood vessels. I would like to demonstrate one case, resulting from a traumatic intraocular hemorrhage, that outlines the vessel walls particularly clearly. The granules were undoubtedly siderotic. They did not bleach out with $KMnO_4$; they were nonbirefringent; and they were unaffected by treatment by Schridde's method for removing formalin artefact. Perle's stain was positive in the immediate vicinity of the granules. The endothelium of the iris vessels and the corneal stormal cells similarly contained siderotic granules.

With siderosis of several months' duration, I was impressed in my cases by the occlusive vascular disease in the retina, and I would agree with the authors' suggestion that many of the abnormal findings in the retina were due to this vascular complication rather than to direct intoxication of the neural tissue.

I might take exception to one analogy which the authors make—that is, the alleged similarity of siderosis to retinitis pigmentosa. In this latter entity, the rods and cones disappear with their respective cell bodies selectively. Unless some other disease process supervenes, the inner layers of the retina are remarkably well preserved in retinitis pigmentosa, the pigment epithelium is only slightly altered morphologically, and the choroid is not usually affected at all. The authors' findings in siderosis are of severe gliosis of the entire retina, fusion of the retina to the choroid, and constant changes in the pigment epithelium and choroid are unlike those of retinitis pigmentosa. On the other hand, they are like the changes seen after cutting off the choroidal and retinal circulation, familiarly seen after nasociliary neurotomy procedures, and are compatible with the thesis that the late changes of siderosis are attributable to vascular occlusion.

This comment lessens in no way the importance of the authors' contributions as regards siderosis, and I found their suggestion that retinal damage from hemorrhage may be a late manifestation of siderosis a most intriguing, as well as a likely possibility.

THE TRANSFORMATION OF CORNEAL STROMAL CELLS TO FIBROBLASTS IN CORNEAL WOUND HEALING*

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Inasmuch as the corneal stroma is avascular and relatively acellular, it provides an especially valuable tool for studying the role of connective tissue cells in wound healing. It is usually stated that the fibroblasts found at the wound edge, and giving rise to the formation of the new connective-tissue fibers, migrate to the wound edge from nests of fibroblasts around capillaries. In wounds made in the center of the avascular cornea migrating cells can easily be observed. Furthermore, it can readily be calculated that fibroblasts cannot migrate rapidly enough from the limbal tissues to provide the source of the fibroblasts found at the wound edge of central corneal wounds. The fastest rate of migration observed for fibroblasts in vivo is 0.29 mm./day,[1] and it would thus require approximately 10 days for fibroblasts to migrate to the center of adult rat corneas, a distance of approximately three mm. Therefore, the fibroblasts found in central corneal wounds must arise from cells already present in the cornea or from cells which migrate at a more rapid rate and transform. For example, monocytes migrating at a rate of 0.35 mm./hr.[2,3] could reach the center of the cornea in eight to nine hours.

In the following experiments the normal pattern of healing in whole mounts of the corneal stroma is described. Evidence is presented to show that corneal stromal cells transform into fibroblasts.

PROCEDURE

Adults rats weighing 250 to 400 gm. were used. The rats were lightly anesthetized with ether (about two minutes) and, using a sterile Graefe knife, a wound approximately two mm. in length was made as near the center of the cornea as possible.

Wounds of 0, 4, 6, 12, 24, 36, 48, 60, 72, 84, 96, and 120 hours were studied. At the indicated experimental time periods the rats were killed with ether and the eyes enucleated and fixed in formalin-alcohol.[4] The epithelium was removed and the stroma dehydrated, cleared and rehydrated for staining as previously described.[4] The tissues were stained for approximately 12 hours in the following diluted solution of Giemsa†: 10 ml. distilled water; 0.4 ml. McIlvaine-Lillie buffer,[5] pH 6.8; 0.1 to 0.3 ml. Giemsa. The intensity of staining varied from batch to batch of Giemsa, and with each new supply of Giemsa solution it was found advisable to do a few preliminary sections at different concentrations of stain.

Following staining, the sections were rinsed in distilled water, and passed for 10 minutes each through four changes of absolute alcohol. This was followed by rinsing for two minutes in 1:1 absolute alcohol-xylol and then two changes of xylol for two minutes each. The sections were left in cedar wood oil‡ overnight, treated again with two two-minute changes of xylol, and mounted in Permount.¶ The sections tended to become bluish during dehydration and clearing but became lavender again when exposed to fluorescent light for a few hours, or upon standing for several days.

RESULTS AND DISCUSSION

During the first 24 postoperative hours the corneal stromal cells at the wound edge un-

* From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University. This study supported by the Knapp Memorial Fund.

† LaMar Giemsa solution.

‡ Fritzsche Brothers, Inc., obtained from A. H. Thomas, Philadelphia.

¶ Obtained from Fisher Scientific Company, New York.

Fig. 1 (Weimar). 0-hour wound. Normal corneal stromal cell. No nucleoli and sparse cytoplasm.

derwent marked morphologic changes and became transformed into fibroblastlike cells. The first noticeable morphologic changes in these corneal stromal cells were (1) an increase in the number of nucleoli per nucleus and (2) an increase in the size of the nucleolus.

Examination of the wound edge (×970) at 4, 6, 12, and 24 hours after wounding showed the gradual transition of the corneal stromal cells into fibroblasts. The first easily discernible increase in the size and number of nucleoli was found at six hours after injury. A steady increase in the size and number of nucleoli occurred at 12 and 24 hours.

Preliminary quantitative studies of the number of nucleoli per nucleus and the diameter of the nucleoli have been made. They indicate that by 12 hours after injury the nucleolar volume per nucleus had increased to approximately 20 times its original value at 0 hours. The nucleolar volume/nucleus continued to increase until 24 hours after wounding when it was about 50 times the original volume. No further increase was noted. These quantitative morphologic analyses will be reported in detail elsewhere. At 120 hours after wounding, the longest time period studied, these large and numerous nucleoli were still present in the fibroblasts. An increase in nucleolar size has been found associated with increased cell metabolism, particularly with intracellular protein synthesis.[6-8]

The nucleus increased in area for the first 24 hours and then decreased to smaller than normal by 48 hours after wounding. At the same time it showed a steady transition from an oval type to a long, narrow type. A marked synthesis of cytoplasm also occurred in these transforming cells during the first 24 hours after wounding, especially between the 12th and 24th hours. At 24 hours the transforming cells had long protoplasmic processes and often appeared stellate in

Fig. 2 (Weimar). 0-hour wound. Normal corneal stromal cell. No nucleoli and sparse cytoplasm.

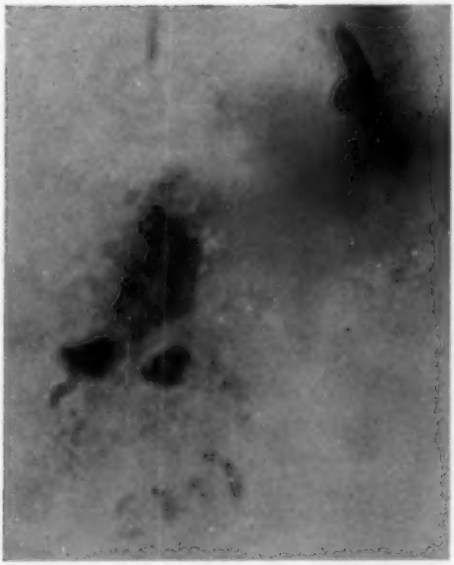

Fig. 3 (Weimar). 12-hour wound. Corneal stromal cell at wound edge with developing cytoplasm and nucleoli.

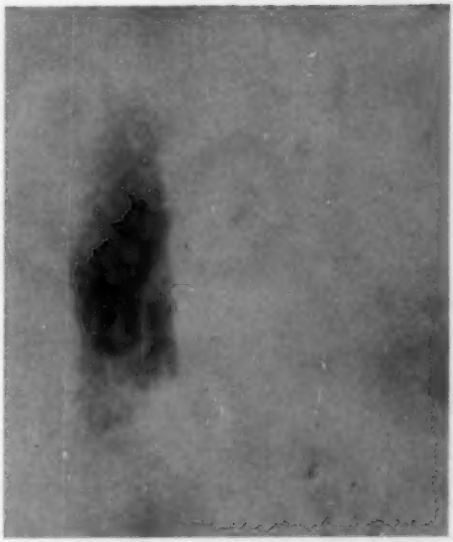

Fig. 4 (Weimar). 12-hour wound. Corneal stromal cell at wound edge with developing cytoplasm and nucleoli.

shape. By 48 hours they were definitely spindle shaped.

These transformations are shown in the accompanying microphotographs (figs. 1-8, originally ×970).

The transformation of cells to fibroblasts was limited to a narrow border about 200 microns wide on each side of the wound edge, as is shown in Figures 9 to 14A, all originally taken at ×430 magnification.

Quantitative cell counts at the wound edge, to be reported in detail elsewhere, revealed that approximately 75 percent of the corneal stromal cells at the wound edge transformed into fibroblasts by 24 hours after injury.

No mast cells were ever found in the normal corneas or in wounded corneas. The mast cells were especially well preserved in this formalin-alcohol fixed material and were observed in large numbers in the limbal tissues. These results agree with the observations of Wassermann[9] who found no participation of mast cells in the healing of tendon wounds.

The only other type of cell found at the wound edge, excluding polymorphonuclear leukocytes, was the monocyte. This in agreement with the studies of avascular healing of the corneal stroma by Pullinger and

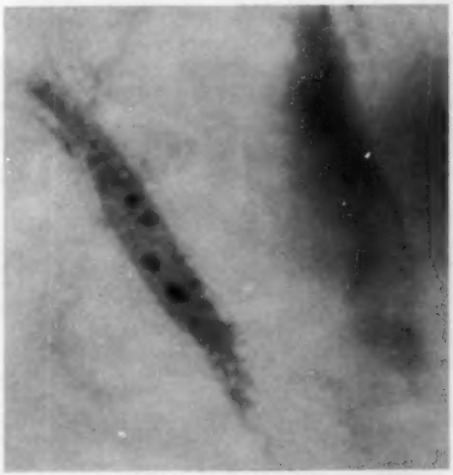

Fig. 5 (Weimar). 24-hour wound. Fibroblast-like cell formed from a corneal stromal cell at the wound edge.

Fig. 6 (Weimar). 36-hour wound. Spindle-shaped fibroblast at wound edge.

Fig. 8 (Weimar). 120-hour wound. Fibroblasts at the wound edge. Note the continued presence of large nucleoli.

Fig. 7 (Weimar). 84-hour wound. Fibroblasts at the wound edge.

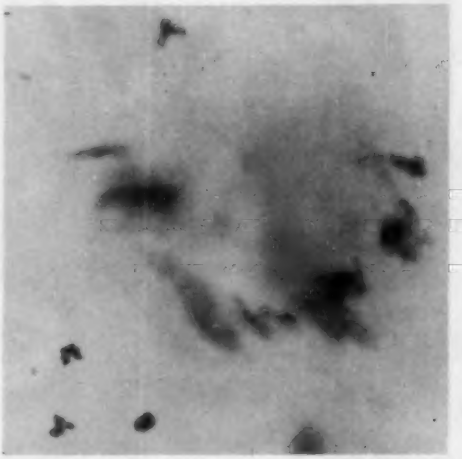

Fig. 9 (Weimar). 12-hour wound. Wound edge. Nucleoli developing in corneal stromal cells. No fibroblasts present.

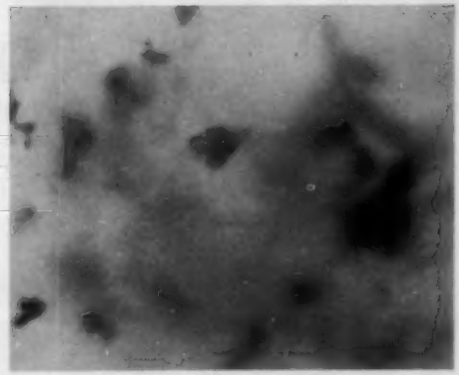

Fig. 9A (Weimar). 12-hour wound. Field immediately adjacent to wound edge.

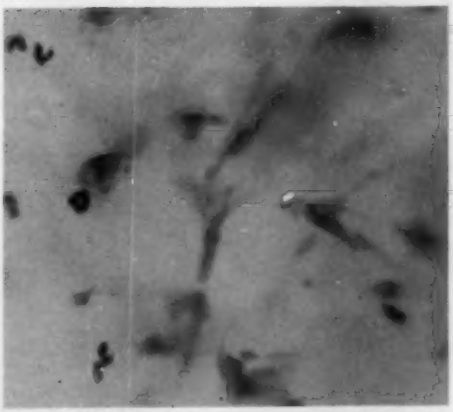

Fig. 10 (Weimar). 24-hour wound. Wound edge with first appearance of fibroblasts.

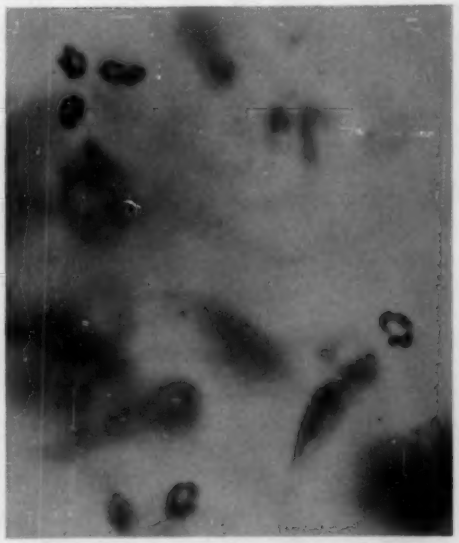

Fig. 10A (Weimar). 24-hour wound. Field immediately adjacent to wound edge. No fibroblasts are seen.

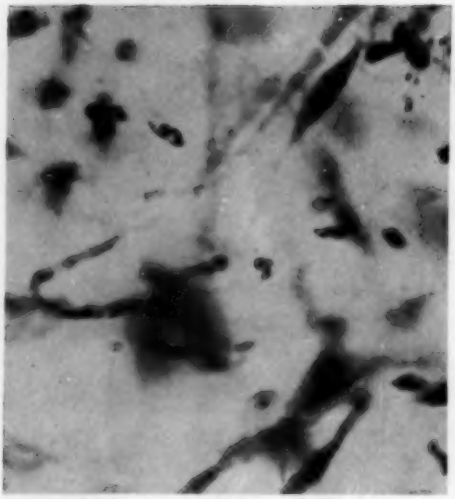

Fig. 11 (Weimar). 36-hour wound. Wound edge with increased number of fibroblasts.

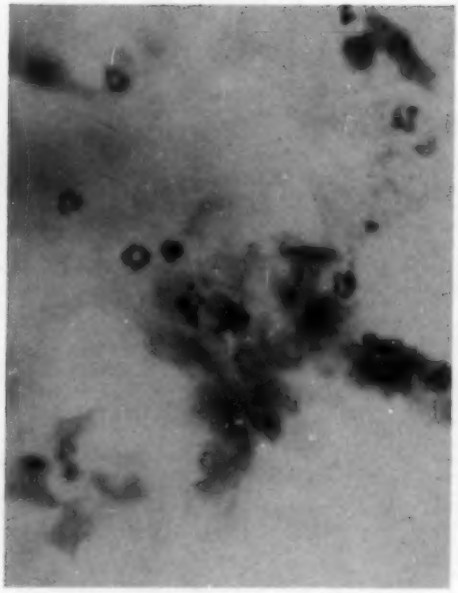

Fig. 11A (Weimar). 36-hour wound. Field immediately adjacent to wound edge. No fibroblasts are seen.

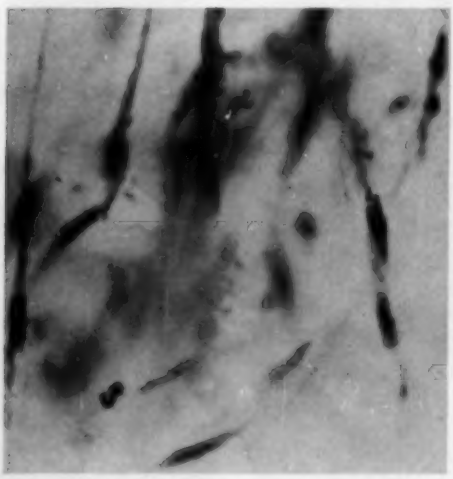

Fig. 12 (Weimar). 48-hour wound. Wound edge with spindle-shaped fibroblasts.

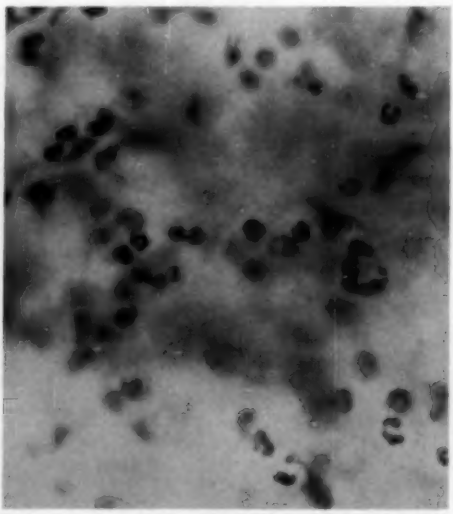

Fig. 12A (Weimar). 48-hour wound. Field immediately adjacent to wound edge. No fibroblasts are seen.

Fig. 13 (Weimar). 84-hour wound. Wound edge with greatly increased number of fibroblasts.

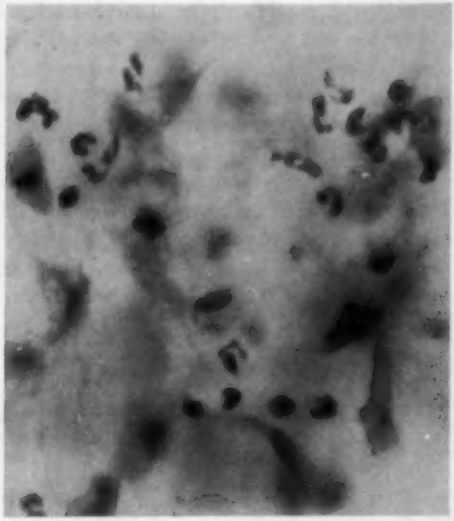

Fig. 13A (Weimar). 84-hour wound. Field immediately adjacent to wound edge. No fibroblasts are seen.

Fig. 14 (Weimar). 120-hour wound. Wound edge. Note that the number of cells has become so dense that cells cannot be distinguished except at the margin of the wound.

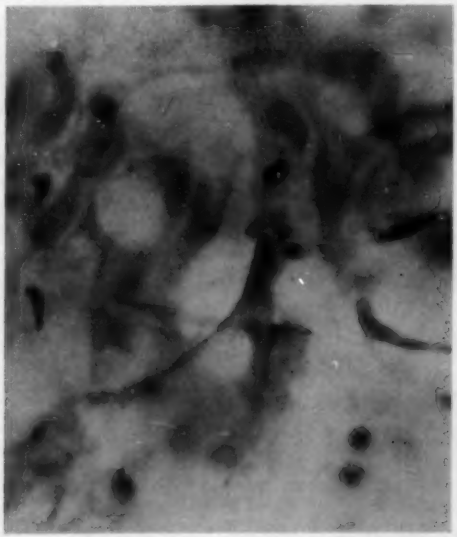

Fig. 14A (Weimar). 120-hour wound. Field immediately adjacent to wound edge. A definite fibroblastlike appearance of cells away from the wound edge can be seen. This effect was not noted at any time period earlier than 120 hours in any of the eyes studied. It is limited to approximately the distance from the wound edge shown in the photograph.

Mann[10] who found only polymorphonuclear leukocytes and wandering cells entered the cornea from the limbal tissues. Fibroblasts and lymphocytes never entered unless blood vessels invaded the cornea. They suggested that the macrophages invading the healing cornea transformed into fibroblasts. Quantitative studies are now being carried out in this laboratory to investigate this possibility and also to determine the role of cell division in supplying the healing wound with fibroblasts.

Summary

The normal pattern of healing in whole mounts of the corneal stroma is described. The corneal stromal cells at the wound edge were found to undergo transformation into fibroblasts within the first 24 hours after injury. The transformation was characterized by the gradual development of large and numerous nucleoli during the first 12 postoperative hours followed by a synthesis of large quantities of deeply basophilic cytoplasm. The transformation of cells to fibroblasts was limited to a narrow border about 200 microns wide on each side of the wound edge.

Mast cells were never seen, except in the limbal tissues, in the normal or in the wounded corneas.

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Discussion

Dr. Alfred E. Maumenee (Baltimore): This detailed study of central corneal wounds by Dr. Weimar is very interesting. I think she has confirmed the observations of others that corneal stromal cells can be replaced by reproduction or cell division of surviving corneal stromal cells.

It has also been noted in other studies that the cornea can be repaired by cells wandering in from the outside of the eye. Several years ago Dr. Walter Kornblueth and I attempted to study this process in corneas in which the stromal cells had been frozen by using an applicator, a rod applicator, that had been reduced to —78°C.

[Slide] With this technique we were able to get a relatively acellular zone of corneal stroma either in the central portion of the cornea or in the periphery, in which we thought practically all the corneal stromal cells in the area had been destroyed.

[Slide] In the central wounds of the cornea we were able to note, very much as Dr. Weimar has just reported, that the normally compact corneal stromal cells became somewhat enlarged in their nucleus and the nucleoli became more evident, and we were able to find mitotic figures in those cells. This was present before any evidence of wandering cells entering from the periphery of the cornea.

However, in other experiments where the lesion was produced at the limbus we thought the repair of the tissue was due in part to macrophages or wandering fibrocytes from the limbus. In animals that had been saturated with trypan blue prior to the freezing experiments, in the central corneal lesion none of these cells contained trypan blue.

[Slide] However, when the lesion was made at

the limbus a great number of cells could be seen with trypan blue granules in them, which we thought indicated that the corneal stromal cell had now come from the wandering cells from outside the cornea.

In using the term "corneal stromal cell" and "fibroblast" in this experiment, I wonder if it is really true and whether we really should say that. Isn't the corneal stromal cell merely a fibroblast? Is there any particular difference in the normal corneal stromal cell and a fibroblast?

In our experiments in which the corneal stromal fibers had not been disrupted, when the cells were repaired in the central portion of the cornea, they were as clear as they had been prior to the entry. Also, in the periphery of the cornea when cells apparently wandered in from the outside, the cornea was as clear as it had been previously.

So, maybe the idea that the fibroblasts bringing in connective tissue different from normal corneal tissue—certainly the cells themselves did not produce this opacity of the cornea—it may be that the changes you are noting in these corneal cells are merely due to edema of the cornea, which allows these cells to appear to be slightly different from the more compact corneal corpuscle in the normally appearing cornea.

Dr. J. Reimer Wolter (Ann Arbor): This is certainly a very fine paper.

Together with Shapiro I have published a very similar study as the present one (Am. J. Ophth., 40:24, 1955). We also found that the corneal stroma cells following injury change into star-shaped cells with a large nucleus. They divide, later become spindle-shaped, and may migrate into corneal areas with cellular destruction. However, we observed that the stroma cells in this stage of reaction and proliferation are still clearly different from immigrating fibroblasts—if the slides are prepared with a histologic technique which really stains both cell types with all their complicated processes.

Therefore, we have to take issue with the statement of Dr. Weimar that she found corneal stroma cells changing into fibroblasts. Using a silver carbonate technique of del Rio Hortega we observed that the corneal stroma cells in a rabbit and man following trauma, operation, and different kinds of chemical injury exhibit a certain fibroblastlike shape. But we were always able to distinguish the reactive form of corneal stroma cells from wandering fibroblasts.

Dr. David Cogan (Boston): To represent the minority party here, may I say the epithelium has not been mentioned as a potential source of replacement cells in the cornea. Twenty years ago such a suggestion would have been accompanied by some sort of penalty. Nevertheless, there are sections through the cornea in which there appear to be all stages in transition of the epithelium into connective tissuelike cells forming a scar seen in rabbits' corneas and occasionally in human corneas.

That concept of conversion of epithelium into connective tissuelike cells is beautifully demonstrated in the lens where we get a metaplasia of lens epithelial cells into connective tissuelike cells. Here there is no question of any foreign invasion such as is always brought up in a question of the cornea and in other tissues. Perhaps Dr. Weimar can tell me it is not like connective tissue but under ordinary light microscopy it does seem that in capsular cataracts the cells and what looks like collagen between the cells have a great similarity to connective tissue.

Therefore, it seems to me that in any comprehensive discussion of the origin of scar tissue in the cornea, the epithelium is also a potential contributing source for the cells.

Dr. Michael Hogan (San Francisco): I would like to ask a question. If one takes a small piece of corneal tissue and puts it in tissue culture, obviously the cells which were damaged at the time of excision of that piece of cornea would show changes similar to those reported here; but on prolonged observation of these cells in culture, they behave exactly as fibroblasts.

I would like to know how Dr. Weimar explains the apparent discrepancy.

Dr. Virginia L. Weimar (closing): First, I would like to discuss Dr. Maumenee's question about the difference in the fibroblasts.

Fibroblasts, whether or not I should say "fibroblasts" or "corneal stromal cells"—in looking at the normal cornea in whole mounts I don't really feel that I can say accurately which is which.

Another observation from work that I am not reporting here today because of the lack of time is that following injury, within the first 12 hours all of the keratoblasts or fibrocytes or histiocytes become macrophagelike and are able to take up neutral red in vitro. So, from then on I could not tell the difference between them. Only in the Giemsa stain material, where I could actually see a monocytelike nucleus, could I really distinguish between types of cells.

As to Dr. Wolter's question that he does not believe the fibrocytes or corneal stromal cells (or whatever we are going to call them) become transformed into fibroblasts, I say that in these preparations of mine there is simply no other source of fibroblasts.

These cells at the wound edge in these preparations do not disappear the way they do in thicker preparations, although I am not prepared to say why. Perhaps it is because in a thicker cornea there is more injury, more activation of destructive enzymes, which destroyed those cells, and then they must be replaced.

However, in this case you have the cells remaining apparently alive; and gradually, if you study them at four hours or six hours or 12 hours, you find that they very slowly change to fibroblastlike cells. A fibroblast at the wound edge at 24 hours must come from some place; yet you cannot detect its appearance in these experiments anywhere between the wound edge and the limbus. Furthermore, the cells which are transforming must be taken into account. At 24 hours the cells which were in the process of transforming are gone and there are

only fibroblasts present—with the exception of a few percent.

I think it is particularly interesting to add that the other source of fibroblasts at the wound edge was probably monocytes. This has been a very much debated question; and although I prefer to believe that monocytes eventually become fibroblasts, I could not find a single piece of research that convinced me it was actually so.

However, Dr. Hulliger in Switzerland has succeeded in showing that monocytes in tissue culture can transform to fibroblasts. This has been shown before, but no one could ever do it under reproducible conditions so that they could do it over and over again, as she did.

In spite of all that, I was not convinced, but she finally convinced me. She has shown that these transformed cells from the monocytes can produce components of collagen, so I think the wandering cells certainly can be claimed to be a source of a part of the fibroblasts at the wound edge.

As to Dr. Cogan's question on the epithelial cells, I would not want to say absolutely "no," but I didn't really see anything that convinced me that they were transforming. I saw some epithelial cells which had large nucleoli, but they always had a different kind of nucleus in color, and the cytoplasm never seemed to become spindle shaped.

As to Dr. Hogan's question about tissue culture, I was very anxious to put these wounded corneas into some kind of controllable situation in vitro. So far I have failed 100 percent because the cells at the wound edge, the cells I have described in this paper, always disappear in a few hours. Even if you put them in when a few large nucleoli have started to develop, after a few hours under any kind of in vitro system I have tried so far, the nucleoli which had developed simply dwindle in size, as if all the metabolism is going on in the other direction.

FURTHER STUDIES ON THE VIABILITY OF FROZEN CORNEAS*

As determined in tissue culture

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Introduction

In a previous communication we described a tissue culture technique for determining the viability of both fresh and frozen corneas.[1] Fresh rabbit corneas were obtained soon after death and explanted in tissue culture flasks and incubated. Corneas which were to be frozen were similarly obtained. Freezing was accomplished by immersing test tubes containing the corneas in a dry ice alcohol mixture (−79°C.). Some corneas were soaked in dilute glycerol solutions prior to freezing as originally described by Eastcott and his associates.[2] Some were frozen without benefit of glycerol soaking. Corneas which were frozen by either method were thawed rapidly in a warm water bath after one hour and then explanted.

The results of this study showed viability of all fresh and glycerol-frozen corneas, with good migration of both epitheliallike and fibroblastlike cells in all cultures at the end of 48 hours. The corneas which were frozen without glycerol soaking showed a lower incidence of viability and evidence of severe cell damage in all cultures. It was felt that this demonstrated that dilute glycerol solutions protected the corneas from the harmful effects of freezing and thawing (table 1).

The present report concerns the effects of certain variables on the viability of glycerol-soaked and glycerol-frozen corneas and is presented in three parts.

Part I. The effects of prolonged storage on glycerol-frozen corneas

This experiment was carried out to determine the length of time corneas could be

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TABLE 1

SUMMARY OF PREVIOUS EXPERIMENT[1]

Corneas in Tissue Culture	Migration	No Migration
Fresh	44	0
Soaked in 15-percent glycerol	60	0
Frozen without soaking*	23	9
Frozen after soaking*	72	0

* Remained frozen only one hour.

frozen and still maintain viability. Such a study would be indicated if one were to employ this technique for long-term preservation of corneal tissue for later transplantation.

MATERIALS AND METHODS

Corneas were removed from adult rabbits under sterile conditions soon after death. The corneas were excised within the limbus so that no conjunctival or scleral tissues were included with the specimen.

The corneas were placed in test tubes containing 15-percent glycerol (USP) by volume in Earle's balanced buffered saline for one hour. The excess fluid was decanted and the test tubes were immersed in a container of dry ice-alcohol slush at −79°C. for three minutes. The test tubes were then placed in screw-top glass bottles and stored in an insulated chest containing solid dry-ice (approximately −79°C.). Dry ice was replenished when necessary to keep each bottle covered to insure a constant temperature.

At various intervals after freezing a specimen was removed from the chest and the test tube was withdrawn from the bottle. The cornea was thawed rapidly by immersing the test tube in a water bath at 38°C. for two minutes. The appearance of the cornea was noted and the specimen was then transported to the Tissue Culture Laboratory where it was explanted and incubated as previously described.[1] Fresh corneas also were explanted to serve as controls.

The specimens were examined microscopically periodically for as long as necessary to determine the growth characteristics. Photomicrographs were taken, and the specimens then discarded.

RESULTS

The results of the present study are shown in Table 2 and detailed in the text as follows:

All fresh control cultures showed evidence of viability within 48 hours. The growth characteristics were essentially as previously described; migration of marginal polygonal cells which resembled epithelium followed by a secondary wave of migration of spindle-shaped cells which resembled fibroblasts (fig. 1).

Corneas which remained frozen for one week were clear on thawing and showed migration of both epithelial cells and fibroblasts after an initial lag period.

Most of the corneas which were frozen for one month were clear on thawing and there was migration of both cell types from most explants. One specimen failed to show any migration and in another no migration of fibroblasts was observed. Changes in the physical appearance were noted for the first

Fig. 1 (Draheim et al.). Extensive migration of epithelial cells and fibroblasts from explant of fresh cornea in tissue culture (×200).

TABLE 2

EFFECTS OF STORAGE ON FROZEN CORNEA

Cornea No.	Duration of Frozen Storage	Tissue Culture Migration		Gross Appearance On Thawing
		Epithelial Cells	Fibroblasts	
9	1 wk.	+	+	Clear
10	1 wk.	+	+	Clear
11	1 wk.	+	+	Clear
12	1 wk.	+	+	Clear
18	1 mo.	0	0	Bubbles on Descemet's membrane
22	1 mo.	+	0	Clear
1	1 mo.	+	+	Clear
3	1 mo.	+	+	Clear
5	1 mo.	+	+	Wrinkled endothelium
7	1 mo.	+	+	Clear
23	7 wk.	+	+	Clear
2	2 mo.	+	0	Wrinkled with bubbles
4	2 mo.	+	+	Clear
6	2 mo.	+	+	Clear
8	2 mo.	+	+	Slight wrinkling
25	4 mo.	0	0	Wrinkled
26	4 mo.	+	+	Clear with some bubbles
27	4 mo.	+	+	Wrinkled with bubbles
28	4 mo.	+	+	Slightly wrinkled with bubbles
13	6 mo.	0	0	Wrinkles and bubbles
15	6 mo.	+	+	Clear
17	6 mo.	+	+	Wrinkled
19	6 mo.	+	+	Wrinkled with bubbles
21	6 mo.	0	0	Wrinkled and slightly cloudy

time. One specimen was wrinkled but viable, another appeared to have bubbles deep in the stroma near Descemet's membrane and was nonviable.

When the period of storage was extended beyond one month the changes in the physical appearance became more marked. This was usually associated with a decrease in the rapidity and extent of migration or an outright failure of migration (fig. 2). Of the five corneas frozen for six months, migration occurred in three cultures. Only one of these was noted to have extensive migration and it was clear when thawed (fig. 3).

PART II: THE EFFECTS OF VARIOUS CONCENTRATIONS OF GLYCEROL ON CORNEAS

When the results of the above experiment were obtained it seemed desirable to define further the problem in an attempt to improve the method of preservation. Attention was focussed first on the soaking solution. Previous reports of tissue preservation by glycerol-freezing have been concerned mainly with 15-percent glycerol solutions[3-4] and our experience had been limited to the use of this concentration. Therefore it was decided to investigate the effects of various concentrations of glycerol on the viability of corneas.

MATERIALS AND METHODS

Rabbit corneas were obtained under sterile conditions as previously described.

The corneas were placed in test tubes containing, 5, 10, 15, 20, 25, 30, 35, and 40 percent glycerol by volume in Earle's saline for one hour. In addition corneas were placed in stock tissue culture medium and Earle's saline to serve as controls. After one hour the specimens were transported to the Tissue Culture Laboratory where they were divided into equal halves. One half of each cornea was fixed for histologic examination and the other half was explanted in a tissue culture flask as previously described.[1]

Fig. 2 (Draheim et al.). No migration from explant of cornea frozen for six months (×200).

Fig. 3 (Draheim et al.). Migration of epithelial cells and fibroblasts from explant of cornea frozen for six months (×200).

Results

The corneas were clear after soaking for one hour in all solutions except Earle's saline. Loss of clarity in this instance was probably due to edema of the cornea for it is well established that corneas will become cloudy when exposed to isotonic saline. The most striking changes occurred in those corneas which were soaked in glycerol-saline concentrations greater than 25 percent. These corneas became thickened and were difficult to cut because of their tough consistency.

Tissue culture explants showed migration from all specimens in this series although there was less extensive migration from those corneas soaked in solutions more concentrated than 25 percent.

Histologic examination in general showed normal stroma in all preparations. There was loosening of the endothelium in some specimens soaked in very dilute glycerol solutions. The most consistent finding was epithelial edema, this being more pronounced in specimens soaked in the higher concentrations.

On the basis of gross and microscopic appearance and tissue culture characteristics it was concluded that 10 to 25 percent concentrations of glycerol diluted with balanced saline caused the least damage to corneas.

Part III: The effects of different rates of freezing on corneas

In a further series of experiments we compared rapid and step freezing after soaking rabbit corneas in various concentrations of glycerol.

Materials and Methods

Rabbit corneas were obtained under sterile conditions and soaked for one hour in the various glycerol concentrations as described above. The corneas were then frozen in the following manner.

The techniques and rates of freezing were predetermined in a separate series of experiments.[5] In these pilot studies platinum-tipped electrodes were placed interlamellarly and temperatures were recorded continuously during freezing (fig. 4). Corneas were frozen rapidly by immersing the test tube directly in the freezing mixture as previously described. A temperature of −70°C. was recorded in less than one minute. Step freezing was accomplished by insulating the test tube containing the cornea until the temperature reached −15°C. Experience showed that this required seven to eight minutes. Removal of the insulator at this point resulted in the two-stage freezing curve as advocated by Smith.[6]

All corneas were thawed rapidly after one hour and then transported to the Tissue Culture Laboratory where they were divided to be explanted and fixed for histologic examination as above.

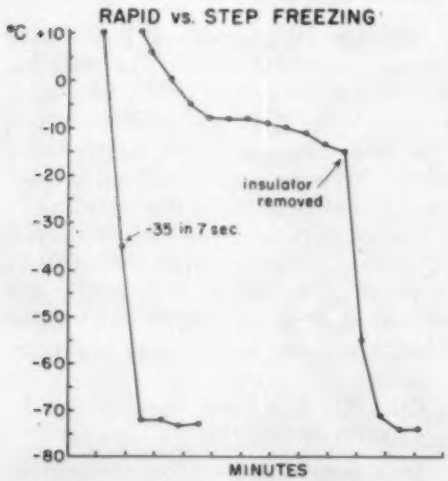

Fig. 4 (Draheim et al.). Examples of freezing curves obtained by the rapid and step freezing techniques.

Results

All rapidly frozen corneas were clear on thawing and there was good migration from explants of tissue frozen after soaking in glycerol concentrations of 10 to 25 percent. All step frozen corneas were cloudy when thawed but the explants showed good migration in the same range of concentrations.

Microscopic sections of both rapidly frozen and step frozen corneas showed marked epithelial changes consisting of edema, bullae formation, superficial necrosis, and in some instances the epithelium was stripped off. Step frozen corneas showed a greater tendency for lamellar separation in the stroma. Changes in the endothelium were variable. In some specimens there was good preservation of histologic detail and in others the endothelium and Descemet's membrane appeared to be stripped off.

Discussion

It is apparent from Part I of this report that prolonged periods of storage result in certain changes in the physical appearance of rapidly frozen corneas. There is an associated decrease in the viability of cells of these corneas as determined in tissue culture. The success of low temperature storage for preservation of biologic materials depends on achieving sufficiently low temperatures to prevent significant molecular transfer and enzyme function. Ice crystal growth in the solid state may take place by growth of large crystals at the expense of smaller at temperatures above −130°C.[7] Occurrence of this phenomenon might explain the reported decay rate of tissue preserved at dry-ice temperatures.

The efficacy of glycerol in protecting tissues from the harmful effects of freezing and thawing has been considered to be due to its ability to prevent formation of intracellular ice crystals by partial dehydration.[4]

This water-binding capacity of glycerol makes less water available to participate in ice crystal formation. In addition, glycerol may afford a "buffering action" in protecting the cells from the hypersalinity that results from ice crystal formation.[8] The most frequently used concentration in earlier studies has been 15-percent glycerol in saline or Ringer's solution. The optimum range for preservation of corneas as judged by migration from the explants in Part II of this report appeared to lie between 10 and 25 percent glycerol in Earle's balanced saline by volume. Concentrations of 30 percent and higher resulted in less extensive migration and evidence of changes in the physical properties of corneas.

In reviewing the available information concerning the effects of rapid and slow freezing on the viability of tissue there is evidence that slow freezing has some theoretic advantages over rapid freezing. Slow freezing initiates formation of large extracellular ice crystals resulting in shrinking of the cells and concentration of the electrolytes in their suspending medium. When the tissue is thawed the cells may assume their original shape but it is suggested that the cell membranes have been damaged by the strong electrolyte solutions.[8, 9]

Rapid freezing initiates the formation of numerous small ice crystals which are distributed uniformly throughout the tissue. Upon thawing there is no evidence of cell membrane rupture but damage to the nucleus and cytoplasm is evident in the frequent vacuolization throughout the tissue.[9]

Smith proposes that tissue damage occurs during cooling before freezing temperatures are reached.[6] To lessen the effects of "thermal shock" it is recommended that cooling proceed at a rate no greater than 2°/min. to −15°C. where maximum concentration of electrolytes begins. After this temperature is reached, rapid freezing is desirable to shorten the time of exposure of the cells to the electrolyte solution.[6] Freezing by this technique results in a two-stage or step freezing curve as obtained in Part III of this report.

The effects of rapid and slow freezing on survival of skin epithelium were reported by Billingham.[10] He found slow freezing superior to rapid when the grafts were frozen without pretreatment. The deleterious effects of rapid freezing were mitigated by soaking the skin in 15-percent glycerol prior to freezing. On the basis of Part III of this report there is no indication that step freezing is superior to rapid freezing in the case of corneas. Pretreatment with glycerol apparently protects the tissue from the undesirable consequences of rapid freezing. If preservation of optical clarity is considered, rapid freezing appears to be the preferred method.

Recently Cockeram, Basu, and Ormsby reported the results of studies concerning the tissue culture viability of frozen rabbit corneas.[11] In these experiments whole eyes were frozen after soaking in glycerol for various periods of time. Corneas which were removed from eyes frozen by their "quick method" and then stored longer than one and one-half hours did not grow when explanted. Failure to maintain viability by this method of freezing may be due to inadequate penetration of glycerol into intact eyes or unpredictable rates of tissue freezing. It is possible that successful preservation of glycerol-soaked corneas by rapid freezing depends upon the use of excised tissue.

Thawing was rapid in all instances in our experiments. If tissue is allowed to thaw slowly there is opportunity for ice crystal growth with resultant mechanical damage.[9] We have observed clouding of corneas which have thawed slowly in the laboratory, apparently due to formation of large ice crystals. It is recommended that thawing be accomplished rapidly by warming in a 38°C. water bath.

Summary and Conclusions

1. The effects of prolonged storage on

glycerol-frozen corneas were evaluated, and it was found that storage of corneas for periods longer than one month resulted in changes in the physical appearance and decreased viability as determined in tissue culture.

2. The effects of various concentrations of glycerol on excised corneas were determined, and it was found that 10-to 25-percent concentrations caused the least change in gross and microscopic appearance and viability as determined in tissue culture.

3. The effects of rapid and step freezing on excised corneas were evaluated, and it was found that although step freezing resulted in clouding of the corneas, variations in the rate of freezing had little effect on viability as determined in tissue culture.

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Discussion

Dr. William H. Havener (Columbus, Ohio): I have enjoyed the opportunity of reading this presentation and also other papers by Dr. Draheim on the subject of corneal preservation. His work has been painstaking and meticulous, and confirms the fact that corneal tissue will remain viable when quick frozen in appropriate concentrations of glycerol. That these preserved corneas will remain clear when transplanted has been demonstrated by Colonel King. Widespread clinical use has not been made of these techniques, and preference has been given to the use of fresh donor material.

Viewed in perspective, corneal transplantation presents the following problems:

Obtaining an adequate supply. This is dependent upon public acceptance of corneal donation. Proper publicity by eye-banks, Lions clubs, and similar related groups is most effective in this respect. It is most important to secure the co-operation of local pathologists and undertakers. To this end, careful attention must be given to cosmetic restoration of the socket with plastic eye caps and molding wax. If enucleation is done with care to avoid traumatizing the extraocular muscles and orbit, discoloration of the lids from bleeding may be eliminated. Should bleeding from the socket occur, no prosthesis should be inserted until bleeding spontaneously stops or the internal carotid is cut (as is done if the autopsy includes a head permit). Disregard of this admonition may lead to swollen discolored lids, difficult for the undertaker to restore.

Adequate methods of preservation of corneal donor material will greatly extend the availability of corneal transplantation. At the present time considerable wastage of outdated corneas occurs, which could be avoided by preservation. It is apparent that Dr. Draheim's work is a real contribution in this area, and will certainly receive clinical trial in the near future.

Maintenance of sterility is a problem which requires further study. Cadaver eyes are almost routinely contaminated. We made a very special effort to scrub and sterilize cadaver eyes before

enucleation in 13 cases. Culture of the bottles in which these eyes were stored showed growth of bacteria in 10 of the 13—indeed, six cases grew Pseudomonas aeruginosa! Use of a variety of antibiotics has been demonstrated to eliminate these bacteria. There is, however, a very slight loss of corneal transparency associated with use of antibiotics. Other methods of sterilization such as ultraviolet or ionizing radiation should be explored. Freezing in glycerol will not destroy bacteria. Fortunately the recipient eye has considerable powers of resistance and I know of several cases of uneventful transplantation following which Pseudomonas was cultured from the donor bottle. Obviously, routine use of antibiotics postoperatively is a reasonable precaution.

Surgical techniques of direct appositional suturing are quite satisfactory. If some refinement of technique could be devised to reduce the high corneal astigmatism so often seen, it would be most welcome.

Maintenance of clarity in the transplant is a problem not yet solved. Cortisone and beta radiation are helpful. Preparation of the cornea with a previous lamellar transplant may permit later placement of a penetrating transplant which will remain clear. Anterior synechias must be avoided by appropriate steps at the time of surgery if at all possible. Immunologic responses to the corneal implant have been suggested as a cause of late opacification. Michaelson has recently demonstrated blood group antigenicity within corneas. It is very likely that the future will bring methods of "typing" corneas, thereby permitting matching of donor and recipient.

I wish to compliment Dr. Draheim on his contributions toward solution of the problem of corneal preservation.

Dr. George K. Smelser (New York): If I may have a few moments, I would like to mention some unpublished experiments carried out by Dr. Richard Copenhaver a few years ago.

Corneas were frozen as described in the present paper and treated with glycerol. Step freezing at very low temperatures, and also slow freezing, was used. The frozen donor corneas were transparent and the transplants were the fully penetrating type. Those which were made by Dr. King, if I recall rightly, were lamellar grafts.

All of the fully penetrating corneal transplants of frozen tissue, in the hands of Copenhaver, failed to produce corneal grafts that maintained their transparency for a clinically useful period. The healing process was usually stormy but there were many instances where he believed he was about to succeed in getting a permanent and fully transparent graft. The best grafts, I should say, could be described as somewhat milky or slightly nebulous.

The cause of the failure is unknown. His technical ability, I think, was good. When he made transplants of fresh normal living cornea, he had excellent grafts, but when the donor tissue had been treated in the manner shown in the present paper to preserve the tissue in a living state, he failed because of some difficulties in the healing process.

All of us here know Dr. Maumenee has had great experience in evaluating techniques in corneal transplants, and it would be very enlightening if we could hear his opinion on why it is that Dr. Copenhaver's fully penetrating grafts did not work, but the lamellar grafts in cats, done by Dr. King, did succeed.

Dr. Jerry W. Draheim (closing): I would like to thank Dr. Havener for his kind remarks. I agree that one must pay meticulous attention to details in all phases of corneal transplantation.

The questions which Dr. Smelser has raised are difficult to answer. My experience with transplantation of these corneas is limited to a few animal procedures which, unfortunately, ended disastrously with pseudomonas infections. We are continuing to evaluate them in another series of operations.

One should be cautious in drawing conclusions from the work presented here today. There is some difference between viability of tissue as determined in vitro and satisfactory biologic acceptance by the host. It has been proposed by some that the stormy postoperative course is due to residual glycerol in the tissue. Glycerol freezing is commonly employed as a means of preserving whole blood. Of course the glycerol must be removed by dialysis before it can be used. We are using a similar technique to remove the glycerol from corneas.

I do not believe that we can cite successful cases of lamellar transplantation of frozen corneas as adequate proof of their viability and therefore suitability for use in penetrating transplants. The biologic problems encountered in penetrating grafts are entirely different from those encountered in lamellar grafts.

A STUDY OF THE UPTAKE OF P^{32}, Zn^{65}, AND I^{131} SERUM ALBUMEN BY EXPERIMENTAL MALIGNANT MELANOMA*

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Failures to demonstrate increased uptake of radiophosphorus by intraocular melanoma, using techniques prescribed, do not apparently all relate to barriers of distance, that is, to the inaccessability of posterior pole tumors. A previous report[1] from this laboratory indicates that the magnitude of radiophosphorus uptake may, even in large melanomas, be insufficient to overcome the disadvantages of location within the globe. Such evidences point to the need for wider understanding of the mechanisms which govern radioisotope uptake by malignant tissues in general and of the factors of metabolism in particular which can explain the differences in uptake encountered among malignancies of the same type.

The purpose of this paper is to report determinations of the radioisotope uptake of experimental malignant melanoma following the administration of P^{32}, RISA (albumen I^{131}), and $Zn.^{65}$

A preliminary phase concerns neutron-activation analyses of the experimental tumor employed in an attempt to determine qualitatively its trace element composition. These analyses were conducted at the Oak Ridge National Laboratory and indicated the presence, in S-91 melanoma, of several metals among which zinc was prominent, being assayed at 236 μg/mg. of tumor (fig. 1).

METHODS

The S-91 Cloudman melanoma was obtained from the National Cancer Institute, Section of Cytochemistry, and prepared as a suspension, 15 gm. in 25 ml. of normal saline. Inbred mice of the DBA strain, male and female, were used in these experiments and the animals fed a standard diet of Purina pellets and tap water, supplemented with fresh greens at four-day intervals. When one ml. injections of tumor suspension were made subcutaneously in the thigh region of each of 108 animals, palpable tumors appeared within six weeks (fig. 2). Of this group, 76 animals were employed in specific uptake studies during the one- to 87-hour interval.

As controls, three groups of eight tumor-bearing mice were injected intramuscularly (1.0 ml.) with non-labelled carrier material which was either 1N H_3PO_4, 1N $ZnCl_2$, or serum albumen: assays of these tumors between one and 72 hours were negative for radioactivity above background levels. Body weights, including tumors, ranged from 17.0 to 30.5 gm. at death.

Radioactive materials of high specific activity were obtained from the Isotope Division, Oak Ridge, Tennessee, as sodium phosphate (P^{32}) and zinc chloride (Zn^{65}), and from Abbott Laboratories as radioiodinated serum albumen (I^{131}). Injections were made intraperitoneally in the case of P^{32} and Zn^{65}, each animal, excepting controls, receiving one ml. of solution containing five micro-

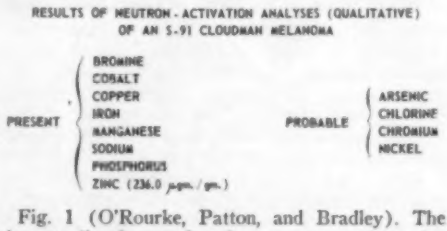

Fig. 1 (O'Rourke, Patton, and Bradley). The elements listed were found on spectrometric analysis of neutron irradiated S-91 melanoma tissue.

* From the Ophthalmology Branch, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare.

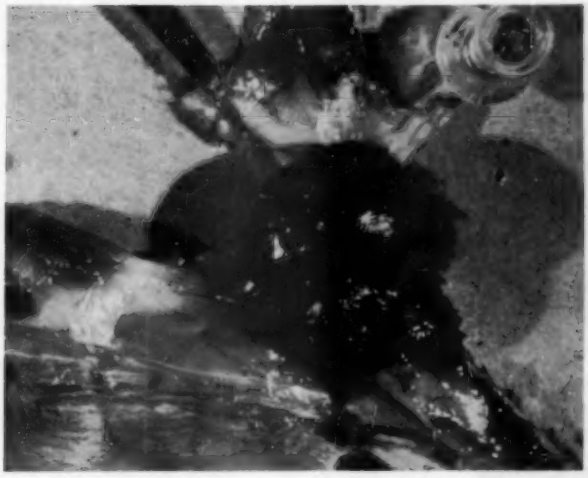

Fig. 2A (O'Rourke, Patton, and Bradley). Example of S-91 melanoma grown on inbred, D.B.A. strain mice; photograph taken six weeks following intramuscular injection of tumor cell suspension into left thigh.

curies per gram body weight. With albumen I^{131} injections were made intravenously and in the same dosage as the other isotopes.

Seventy-six of the tumor-bearing mice used in these experiments received injections of labelled material a few days after all melanomas had become palpable and firm, and groups of them were killed at various intervals between one and 84 hours after the injections, along with 32 controls.

The animals were killed by Nembutal® injection and the tumor mass removed by blunt dissection, care being taken to debride extraneous tissue; between 2.1 and 10.6 gm. wet weight of melanoma tissue was obtained from each animal.

In these determinations tumor masses were digested with fuming nitric acid, brought to a 100-ml. volume with water and the radioactivity of a 1.0-ml. aliquot measured, using a well-type scintillation counter. Sample counting times were sufficient to give a probability error of ± 1.0 percent of the total count.

Because preliminary studies indicated the hepatic uptake of each isotope to be higher than other body organs, liver samples were selected for similar analysis to afford a basis for comparison with tumor values (fig. 4).

Tumor radioactivity was recorded as Differential Absorption Ratio in order to express uptake as a fraction of the administered dose per gm. body weight, the value being derived as follows: μc./gm.B.W.:1 = μc./gm.tumor:DAR. All calculations were corrected for the half-life decay rates of

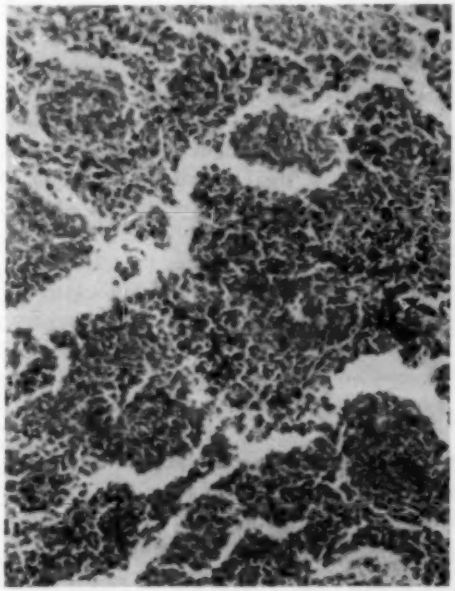

Fig. 2B (O'Rourke, Patton, and Bradley). Photomicrograph, low power, of S-91 melanoma, showing density of malignant cells.

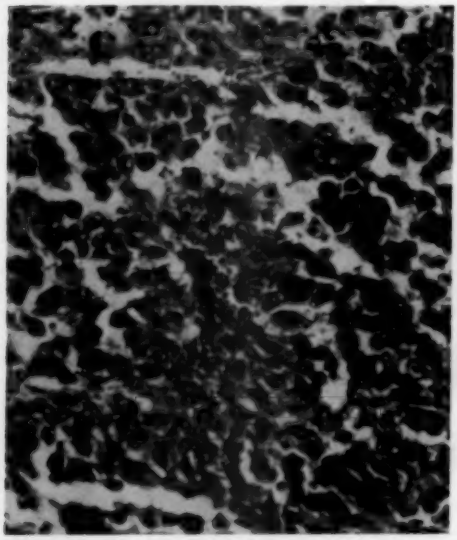

Fig. 2C (O'Rourke, Patton, and Bradley). Photomicrograph, high power, of S-91 melanoma showing evidence of malignant change.

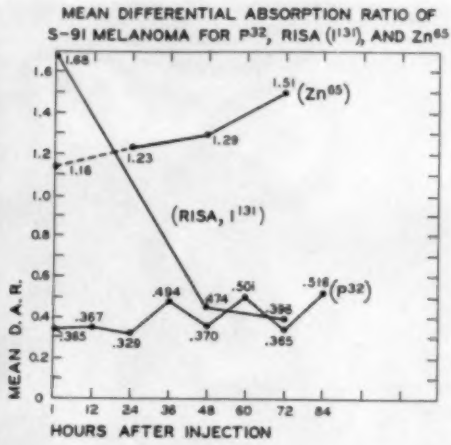

Fig. 3 (O'Rourke, Patton, and Bradley). Expressed as Mean Differential Absorption Ration, the S-91 melanoma uptake of Zn^{65} and RISA (I^{131}) exceeds that of P^{32} at one hour postinjection, following which Zn^{65} levels remained elevated while RISA (I^{131}) values fell sharply.

the isotopes employed (P^{32}, 14.3 days; I^{131}, 8.14 days; Zn^{65}, 250 days).

Results

In Figure 3 are depicted graphically the magnitudes of difference in the mean DAR values of S-91 melanoma for the three radioactive sources studied and what they indicate principally is that the initial uptake of Zn^{65} and RISA (I^{131}) greatly exceeds the levels attained using P^{32}. Beyond 24 hours, however, the tendency is that the mean DAR of Zn^{65} and P^{32} will vary within comparatively narrow limits while corresponding values for RISA (I^{131}) show an approximate 60-percent reduction from the one-hour level.

Differences both in individual tumor weights and in their relations to total body weight are indicated in Table 1: from this data there emerges little evidence that, under conditions of this study, melanoma size is related to specific (μc./mg.) uptake of radioactive materials. Wet tumor weights ranged between 2.17 and 11.8 gm. and the distribution of DAR values appeared to vary randomly among the various gram weight classes, though probably the number of tumors assayed for Zn^{65} and I^{131} content are in these instances too small to sustain this impression.

HEPATIC UPTAKE (DAR)*

	P^{32}	Zn65	I^{131}
1 hour	1.632	3.64	0.770
24 hours	0.543	3.23	0.328
48 hours	0.326	1.40	0.435
72 hours	0.148	1.41	0.143

* Each value = mean DAR of five samples

Fig. 4 (O'Rourke, Patton, and Bradley). The magnitude of hepatic uptake expressed as Mean Differential Absorption Ratio, derived from 60 specimens, was similar to corresponding values representing S-91 melanoma uptake of P^{32}, Zn^{65}, and RISA (I^{131}).

TABLE 1

DATA ON UPTAKE OF RADIOACTIVE PHOSPHORUS (P^{32}), ZINC (Zn^{65}) AND IODINATED ALBUMEN (I^{131}) FOR 76 MOUSE TUMORS

	Body Weight (gm.)	Tumor Weight	Tumor DAR
P^{32}			
(hr.)			
1	21.1	3.7	0.290
	21.3	4.9	0.363
	25.7	5.8	0.612
	21.8	3.1	0.356
	22.5	3.9	0.194
	17.0	4.5	0.376
12	23.8	8.1	0.296
	25.3	8.7	0.486
	30.5	10.6	0.390
	24.0	8.0	0.293
	25.1	7.5	0.413
	25.1	8.6	0.329
24	20.1	4.2	0.303
	23.2	5.5	0.336
	21.1	6.0	0.289
	21.0	5.9	0.313
	23.1	5.9	0.446
	24.3	3.6	0.289
36	18.1	10.8	0.140
	24.1	7.4	0.629
	20.3	5.2	0.456
	23.6	9.3	0.559
	23.0	7.5	0.524
	22.8	8.2	0.661
48	24.0	11.8	0.236
	23.6	4.7	0.423
	19.8	6.6	0.472
	23.1	6.1	0.379
	22.8	5.6	0.502
	22.1	3.2	0.209
60	25.0	8.9	0.580
	23.8	8.7	0.436
	24.2	8.4	0.489
	23.4	7.5	0.468
	23.8	7.5	0.474
	28.9	8.2	0.561
72	19.3	3.9	0.151
	23.1	5.6	0.483
	21.5	4.1	0.329
	25.0	6.2	0.440
	26.1	7.5	0.487
	20.7	5.5	0.304
84	21.7	6.7	0.362
	25.5	10.2	0.487
	22.1	6.8	0.517
	19.8	0.6	0.698

TABLE 1—*continued*

	Body Weight (gm.)	Tumor Weight	Tumor DAR
Zn^{65}			
(hr.)			
24	25.7	8.1	0.264
	22.6	7.1	1.36
	26.4	8.0	1.30
	28.6	9.4	1.26
	26.6	8.8	1.44
	23.7	3.7	1.78
48	22.3	6.9	1.78
	24.6	8.5	0.534
	25.2	9.0	0.192
	19.6	5.6	1.98
	22.1	7.1	1.26
	21.3	6.0	2.0
72	27.9	10.7	1.84
	24.1	8.3	1.52
	24.0	5.8	0.56
	23.0	4.3	2.00
	26.0	9.0	1.65
	24.2	6.72	1.63
RISA I^{131}			
(hr.)			
1	26.0	9.32	1.87
	25.5	2.17	2.03
	26.48	7.63	2.10
	27.82	8.62	0.720
48	31.2	10.9	0.260
	28.3	9.8	0.780
	27.1	7.8	0.512
	27.8	7.7	0.346
72	26.9	8.1	0.132
	29.1	9.9	0.482
	26.1	9.0	0.762
	25.8	8.0	0.210

Regarding hepatic uptake it was noted here, as it has been in previous studies, that for most isotopes liver DAR is highest among the body organs, serving thereby as the norm in determining, within broad limits, what levels, in other body tissues, indicate high specific uptake. In general terms melanoma DAR figures for the three sources were within range of corresponding hepatic values (fig. 4) indicating that the melanoma affinity is above body average for each of them.

In reviewing the magnitude of specific uptake changes in time, P^{32} results appear, in a statistical way, to be much more secure than are the fewer data concerning Zn^{65} and RISA (I^{131}): 46 animals were assayed for

P^{32} uptake at eight intervals and their tumor DAR values lay between the rather narrow limits of 0.194 and 0.612.

While using Zn^{65} there occurred a greater variation in individual melanoma DAR, this trend does not obscure the fact that overall Zn^{65} uptake is higher than corresponding values for P^{32} during the one- to 72-hour interval.

Most notable of the impressions derived from using radioiodinated serum is the initial high uptake recorded at one hour, actually the highest among all the tumors studied. Since no attempt was made to prevent thyroid uptake of the degraded RISA as, for example, by preliminary administration of iodine, it remains possible, in this way, that the fall noted by 48 hours might be modified.

Standard deviation in DAR values were lowest in the P^{32} series, ranging between 0.047 (60 hours) and 0.172 (12 hours): corresponding values for Zn^{65} were 0.459 (72 hours) and 0.713 (48 hours) and for RISA (I^{131}) were 0.198 (48 hours) and 0.560 (one hour).

Differences in tumor DAR were at the one-percent level of significance in all instances excepting the 48-hour interval between P^{32} and Zn^{65} (two percent) and between Zn^{65} and RISA (I^{131}) (five percent), and at the 72-hour interval between Zn^{65} and I^{131} (two percent).

Discussion

In that the present work is quite preliminary its main conclusion, that melanoma takes up less P^{32} than Zn^{65} or RISA (I^{131}), will not sustain a complete discussion of the several mechanisms that are possibly involved.

In general, the tendency of some malignancies to take up relatively greater amounts of P^{32} than do their normal tissues of origin is explained by the increased amounts of nucleic acid that are synthesized by rapidly growing cells.[2] How this generalization applies to the uptake by various tumors and to different stages of the same tumor is not known, but probably it is unwise to assume that each component of the tumor cell is altered by the various neoplastic processes in the same way in all types of malignancy: this considered, there seems little assurance that melanoma will reproduce the P^{32} uptake pattern observed in other types of tumor and the implication is that melanoma deserves separate study.

An additional factor of significance for the problem of P^{32} detection is the variation in composition and population of tumor cells that apparently occur among malignancies of the same type. Since the proportion of nucleic acid in various cancer cells may change at various stages of growth[3] and, in mitosis, may fail to keep pace with the rate of cell division,[4] it is reasonable to expect that variations in P^{32} uptake among the melanomas, seen clinically, will occur. The ancillary observation of Casperson,[5] that in epithelial tumors the nucleic acid content is higher in peripheral cells, suggests an unequal distribution of that material among malignant cells.

With regard to cell population among tumors of a particular type, it is possible for the proportion of tumorous cells found in certain epithelial malignancies to vary between 10 and 90 percent[3] indicating the need to control this factor by microscopic criteria, as suggested by Chalkley.[6] An example of this is the method by which differences in the cytochrome oxidase content of certain thyroid adenomas were resolved by corrections for variations in cell content.[7]

Since changes in composition and weight of tumor cells are more frequent among primary malignancies, arising from normal tissues of origin, homogenity may be improved by the use of experimental tumors,[8] transplantation of which tends to concentrate cellular elements without marked alteration in chemical behavior. Collation of specific tumor affinity for various isotopes under these experimental conditions is then more standardized than in clinical studies, which fact may explain the observation that P^{32} uptake by

these tumors was much less variable than with primary ocular malignancies previously reported.[1]

The characteristic of malignant tissue to elicit from the host a constant new growth of capillary endothelium causes an increased vascularity of the tumor bed[8,9] which is one explanation offered for the increased uptake of certain blood borne isotopes. The use of RISA (I^{131}) is principally based on this reasoning in that it persists at relatively high blood levels a great deal longer than most other chemical compounds, while not concerning directly the intracellular metabolism of the cancer cell. Opposed to the maintenance of high blood levels of RISA is the factor of thyroid uptake of free I^{131} and the rapid fall of tumor and liver activity after 24 hours is probably directly related to this phenomenon.

While the uptake and retention of Zn^{65} exceeds that of P^{32} in both liver and tumor the reasons for it are, at the present time, largely unexplained. Apart from the presence of zinc in carbonic anhydrase[10] and its probable association with glycolysis and insulin reactions, little is known about the metabolic activities with which this metal is associated. Evidence exists that at least in some tissues zinc is associated with metabolism in cell nuclei[11] which finding may corraborate recent evidence that labelled insulin (I^{131}) tends to localize intracellularly in liver, kidney, and muscle.[12]

While the zinc content of normal tissues has been widely studied that of neoplasms has been compared with normal values in only a few instances.[13–15] Woods showed that the glycolysis rate in melanoma slices associated with use of a zinc deficient insulin was greatly potentiated by the subsequent addition of free zinc.[16] The observation that mammary gland tumors take up and retain considerably more zinc than does normal breast tissue[13] is one of the few evidences that malignant tissue metabolism utilizes this element; another is the prominent zinc level observed in the preliminary experiments recorded here, concerning neutron-activation analysis of experimental malignant melanoma.

When malignant change occurs, the associated increase in rate of glycolysis represents an alteration, which is likely more constant and of greater magnitude in cell chemistry, than are the others that concern nucleoprotein synthesis and blood supply. Should the uptake of Zn^{65} be in proportion to the intensity of local glycolysis, its use might permit a favorable tumor/normal tissue contrast for localization studies, there being indications that glycolysis takes place at a very intense rate within the cell nucleus.[17]

The evidence that a number of metals, zinc included, may combine with natural melanin complexes is well documented[18] and makes it necessary to consider that the high uptake of Zn^{65} by melanoma may be a phenomenon which is specific to pigmented tissues, irrespective of malignancy. This work further indicates that the highest known concentrations of zinc in living matter occur in the melanin pigmented tissues of the eye of some fresh water fishes,[19] and that the amounts encountered in corresponding tissues of mammals, while a good deal lower, always exceed those found in unpigmented tissues of the same animals.[20]

In the practical sense there are some limitations to use of Zn^{65} clinically: Localization is difficult because of scatter of its high energy gamma emissions (1.12 m.e.v.) and use of a less energetic form (Zn^{69}) is hampered by production difficulty and short half-life.

Conclusions

1. Neutron-activation analyses of experimental malignant melanoma (S-91 Cloudman) reveal the presence of trace amounts of zinc in μg. quantities plus several other metals.

2. When isotope uptake studies were carried on this tumor using P^{32}, Zn^{65}, and RISA (I^{131}) albumen in 76 tumor-bearing mice, results indicated that initially the tu-

mor Differential Absorption Ratio for P^{32} is decidedly less than the corresponding values for the other two isotopes.

3. Tumor content of RISA (I^{131}) fell rapidly after one hour when thyroid uptake of the iodine was not prevented while levels of P^{32} and Zn^{65} remained closer to the initial (one hour) values, the Zn^{65} content being about three times greater than P^{32} at the intervals tested (one to 72 hours).

4. Limited discussion of the metabolic patterns responsible for these results concerns three main points:

a. That the inconsistency of the P^{32} uptake by tumors encountered clinically may, among other factors, be due to the variations of cell population and composition found in primary malignant tumors.

b. That high initial tumor levels of RISA (I^{131}) reflected tumor vascularity and were probably reduced subsequently by thyroid gland uptake, preliminary saturation of which was omitted.

c. That sustained high levels of zinc uptake by melanoma may reflect either intensity of tumor tissue glycolysis or specific affinity of melanin for certain metallic ions.

Ophthalmology Branch (14).

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Discussion

Dr. Edwin Dunphy (Boston): I shall only attempt to discuss this interesting paper and its possible relation to clinical ophthalmology.

Dr. O'Rourke and his associates have amply demonstrated that the S^{35} mouse melanoma takes up less P^{32} than Zn^{65} and I^{131}, especially shortly after injection. In the body of the paper they point out the fact that it is unfortunate that there are certain limitations in the use of his isotope of zinc in ophthalmology because of the scatter of high energy gamma particles.

They have advanced certain possible explanations why the P^{32} uptake may vary during different stages of tumor growth, and between tumors of the same cell type. Most of us who have had experience with the P^{32} test in ophthalmology will agree that similar differences are seen clinically, which is somewhat analogous.

For instance, some malignant melanomas of the choroid will show perhaps a 40-percent differential uptake as compared with the other eye, while others of apparently the same size and equally accessible to the Geiger counter will show 150-percent difference. In each case the patient had received the same amount of P^{32} per kg. of body weight, and the test was performed in the same 24-hour interval.

In my own experience no false negatives have occurred in choroidal tumors when the lesion was accessible to the counter. It is perfectly possible, of course, that this may happen during an inactive phase of the tumor metabolism, and for this reason I have always placed much more reliance on the positive test than on a negative one.

In their bioassay of this experimental tumor the authors record the isotope uptake as its differential absorption ratio, that is, the relatoinship of the content of the isotope in microcuries per gm., to that of the whole body weight. Although their results indicate that this differential absorption ratio is relatively small, I do not think this is of too much importance in the practical application of the test.

What is important to the clinician is the uptake of the tumor compared to its surrounding normal tissue. The neurosurgeons, for instance, have found P^{32} good in delineating brain tumors at operation, not because the tumor takes up much of the isotope (it does not) but because the normal brain takes up so little.

Likewise, in the human eye a malignant melanoma of the choroid or ciliary body expanding into the vitreous and covered over by sclera, both of which tissues show relatively little concentration, should show an increased uptake in relation to its background. It is true, however, that the normal choroid shows a good concentration of its own, and this may confuse the picture.

Some years ago Selverstone and I performed radio-assay of the various ocular tissues extracted from enucleated eyes following intravenous injection of P^{32}. The following slide will demonstrate our findings.

[Slide] This is what we call the activity ratio compared to that of the cornea, which was found in every case to be very low, and we have indicated the radioactivity of the cornea in all these cases as one, as the standard, and the other figures are in relation to the cornea in each particular case.

You will see that the sclera, reading across from left to right, has a relatively low uptake compared to the cornea. The aqueous is very low; so are the vitreous and the lens. The iris and ciliary body are definitely higher. The choroid is again higher, and please note this case on your extreme right. The retina has somewhat of an uptake, and the optic nerve more.

Patient W. and Patient H. had malignant melanomas which were analyzed. In the case of W., notice the activity of the tumor compared to that of the same patient's choroid. You see it is perhaps two and one-half times as much, whereas in the case of H., on the extreme right, the activity of the tumor is actually less than that of the choroid.

This is confusing, of course, and it is for that reason that I stopped testing back in 1949. I thought we really would not be able to apply this test clinically because of the rather high uptake rate of the normal choroid itself.

However, as the years have gone by, my associates and I have found that the test does have some practical value, and I think it is due to the fact that the tumor really is expanding into areas like the vitreous and those covered by the sclera, both tissues having so little uptake of their own.

ANTERIOR CHAMBER PERFUSION STUDIES*

II. Controlled particle size in relationship to pore size

Philip A. Peter, M.D., Wood Lyda, M.D., and Narendra Krishna, M.B.
Seattle, Washington

The purpose of the present study was to determine the effect of uniform size latex particles on the resistance of the chamber angle. It was felt that if the porosity of the intratrabecular spaces is heterogenous, particles of small diameter should only block a fraction of the pores and that larger diameter-particles should obstruct a greater portion of the outflow channels. It was hoped that a correlation could be established between latex particle diameter and the trabecular pore size as manifested in the change of resistance to outflow. It was further hoped that microscopic studies would indicate at what point blockage to outflow occurred, as well as the size of particles that would or would not pass through the trabecula and collecting channels. Since hyaluronidase reduces the resistance to outflow, a correlation between latex particle size and the hyaluronidase effect was also considered.

The early work of Leber and the recent work of Theobald, Friedenwald, Thomassen, and Bakken has established that aqueous veins have their origin in Schlemm's canal and that there exist direct channels from the outer wall of Schlemm's canal to the episcleral venous plexus. The clinical detection of aqueous veins by Ascher and Goldmann and the Neoprene casts of Ashton demonstrate the communications between Schlemm's canal and the scleral surface of the eye.

Leber, Schwalbe, and Maggiore felt that under normal conditions the canal of Schlemm was not in direct communication with the intratrabecular spaces and that the aqueous entered Schlemm's canal by filtration. However, the presence of pores or direct channels from the anterior chamber through the trabecula into Schlemm's canal has been suggested by the regurgitation of red cells into the anterior chamber by Kronfeld and confirmed by the histologic studies of Theobald, Sondermann, Ashton, Thomassen, and Bakken.

Huggert demonstrated that intracamerally injected bacteria of approximately 1.5 μ could be collected from the aqueous veins in enucleated rabbit eyes and that bacteria up to 2.5 μ would pass through these channels in the human eye.

François et al., utilizing a microradiographic technique, showed that particles less than 1.5 μ would pass through the trabecular meshwork whereas particles greater than 2.5 μ would not pass into Schlemm's canal in the rabbit eye.

In an ingenious study, Huggert collected chromium phosphate particles from the ligated aqueous veins of eyes previously injected intracamerally. When the filtrate was studied by optical and electron microscopy a distribution curve established that the greatest concentration of particles was approximately 1.1 μ in size in rabbits and 2.25 μ in humans. In rabbits the majority of pores were within the 0.1 to 1.0 μ range and, of these, the smaller size openings markedly predominated.

The studies of Huggert and François et al. have adequately established the maximal sized particles that will pass through the trabecula and outflow channels. However, heteroporosity of the intratrabecular spaces and the relative predominance of certain size openings has only been suggested by the work of Huggert. Therefore, the present study is an attempt more accurately to an-

* From the University of Washington Medical School. Supported by funds through Grant B-845 of the National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Maryland.

alyse the distribution of pore sizes within the outflow channels of the eye.

The perfusion technique of Bárány for determining the facility of outflow is simple and easily applied to controlled studies. Becker has established a close correlation between perfusion in vitro and in vivo and the tonographic method of determining facility of outflow. The present investigation is an attempt to produce varying degrees of physiologic blockage of the anterior chamber angle by intracameral injection of controlled size particles and to correlate these changes with the alteration in resistance to outflow as measured by perfusion.

Method

For the purpose of the present study a perfusion apparatus, modified after Bárány, was employed and was similar to that utilized in our previous studies.*

Two one-ml. pipettes, calibrated in 0.01 ml., were placed horizontally and at the same level as the eye to be perfused. The tip of the first pipette was connected by a short length of intravenous latex tubing to the base of a three way stop-cock and the second pipette was connected to its side arm. The outlet of the stop-cock was adapted to a one-half inch 25-gauge stainless steel needle.

Saline buffer solution was drawn into the stop-cock and one pipette, then the buffered latex solution was injected through the rubber tubing of the second pipette. In this manner the solutions in the parallel pipettes were contiguous with each other being separated only by the internal wall of the stop-cock. When a steady state with the saline buffer was reached, the stop-cock was turned and the buffered latex solution then allowed to enter the eye without trauma. To the open end of the pipettes was adapted a manostat of water regulated to a pressure equivalent to 20 mm. Hg. The pressure was exerted equally to each pipette.

TABLE 1

Uniformity particle size of mono-dispersed latexes

Particle Diameter	Standard Deviation	Number of Determinations
μ	μ	
0.138	0.0062	526
0.264	0.0060	577
0.365	0.0079	438
0.880	0.0080	1164
1.171	0.0133	315

The saline buffer solution used in the experiments consisted of nine parts physiologic solution and one part Sorenson's phosphate buffer adjusted to pH 8.04. The latex particles† utilized were mono-dispersed latexes' particles of unusually uniform diameter, as shown in Table 1.

The latex particles were suspended as a five-percent concentration in the saline buffer. All solutions and the eyes were maintained at 25 to 27°C.

Albino rabbits, approximately 2.5 to 3.5 kg., were used in this study. Upon killing the animals by an intravenous air injection, the eyes were enucleated immediately and excessive conjunctiva excised. The eyes were placed in a Petri dish with saline-moistened cotton. Canalization of the anterior chamber by the needle was made approximately two mm. anterior to the limbus in an oblique direction. In the present method only one puncture of the anterior chamber was required and the "massage" effect was eliminated. The actual perfusion usually was initiated within 15 minutes after enucleation.

Perfusion was performed on at least 10 enucleated eyes with each particle size studied. The time in minutes required for 0.01 ml. of solution to enter the anterior chamber is a measure of the resistance to flow in the enucleated eyes.

As the latex particles filled the angle, the resistance increased. The rate of increase in resistance was directly proportional to the

* Unpublished report.

† Dow Chemical Company, Midland, Michigan.

Fig. 1 (Peter, Lyda, and Krishna). Tracing of perfusion. Latex diameter, 0.880μ.

rapidity of gross angle blockage. However, the rapidity of angle blockage was not related to the size of the latex particle utilized. In almost every eye the latex particles formed a zone encircling the chamber angle. As the latex ring approached a complete encircling of the angle, that is, macroscopically complete angle blockage, the resistance increased rapidly and soon thereafter a steady state was reached (fig. 1 and 2).

Results

Because of its distinct color characteristics, the first appearance of the latex particles within the anterior chamber was readily apparent. The "dead" space from the center of the stop-cock to the anterior chamber was approximately 0.03 ml.

The filling of the anterior chamber with the latex particles may be compared with that of snow "banking" against a fence. Usually either one portion of the angle filled first and then the contiguous portions filled until the entire angle was blocked or a series of "pseudopods" stretched toward the angle, where "banking" occurred in both directions until a ring of latex was formed completely blocking the angle macroscopically. Only after the blocking of the angle did the central portion of the anterior chamber fill.

Latex particles of 1.17 μ could not be maintained in solutions diluted to five percent or less unless the surface tension was changed by additives. Since the perfusion solution is different, the results with 1.17 μ latex particles cannot be used in the correlation and will be reported another time.

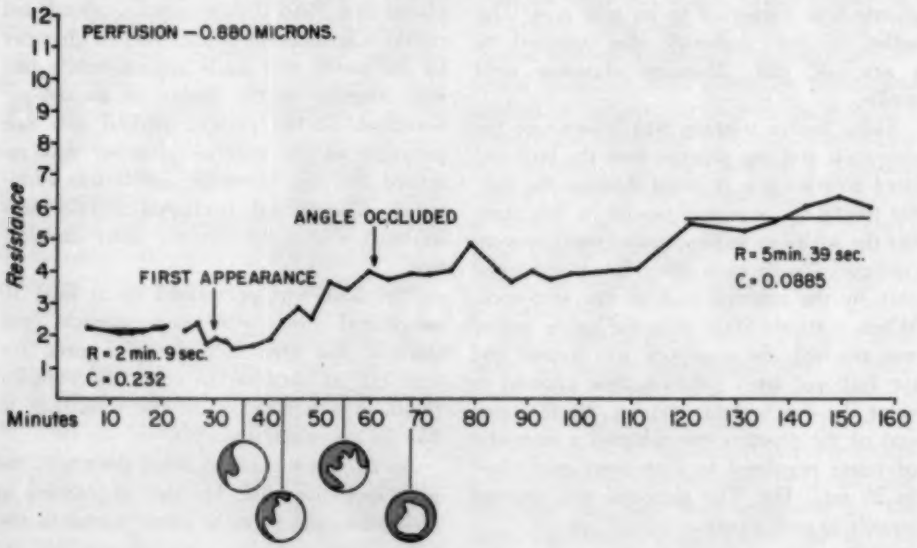

Fig. 2 (Peter, Lyda, and Krishna). Tracing of perfusion. Latex diameter 0.880μ.

TABLE 2

RESULTS—PERFUSION 43 ENUCLEATED RABBIT EYES

Particle Size	No. of Eyes	Average Resistance Buffer		Average C of Buffer	Average Resistance Latex		Average C of Latex
(mμ)		(min.)	(sec.)		(min.)	(sec.)	
0.138	10	2	41	0.1869	7	7	0.067
0.264	10	2	38	0.1895	9	7	0.056
0.365	10	2	33	0.1960	5	38	0.083
0.880	13	2	26	0.2121	7	10	0.072

There was essentially no difference in the time required for one ml. of saline buffer solution or for one ml. of buffered latex solution to flow through the apparatus itself.

Previous studies of our own and those of others have established a normal for the facility of outflow in perfusion of enucleated rabbit eyes. In this study, the average resistance of the 43 control eyes was two minutes and 34 seconds, or, a facility of outflow of 0.195. For the purpose of this study it was elected that each eye should serve as its own control as the steady-state of the saline buffer was compared with the steady state of the latex. Table 2 illustrates the average resistance and facility of outflow for each of the four particle sizes utilized in this study.

A plot of the ratio of $\frac{\text{C-Latex}}{\text{C-Buffer}}$ shows the wide scatter of these points (fig. 3). The letter "X" identifies the position of the average ratio for each particle size studied. It may be further noted that the average ratio for each particle size studied lie closely together and are of no significance.

An analysis of the variance of the ratio of $\frac{\text{C-Latex}}{\text{C-Buffer}}$ between the groups and also within each group shows an "F" of 1.67, whereas, an "F" score of approximately 14.6 is required to be significant at the five-percent level of confidence. Statistically, then, there was no significant difference in the resistance of the chamber angle when a physiologic blockage of the outflow channels was produced by controlled sized particles having a size range from 0.138 μ to 0.880 μ. That is, particles of a size range that should pass through the trabeculum have similar resistance values.

MICROSCOPIC STUDIES

Histiologic studies were made of the perfused rabbit eyes at the completion of each experiment. Since the latex particles are lost with the usual fixation and staining techniques the eyes were cut in frozen section and stained with aqueous eosin and hematoxylin without clearing. They were then mounted on the slide in a gelatin film. Sections were made for each of the five particle sizes used in the experiment and studied with regular and polarized light microscopes.

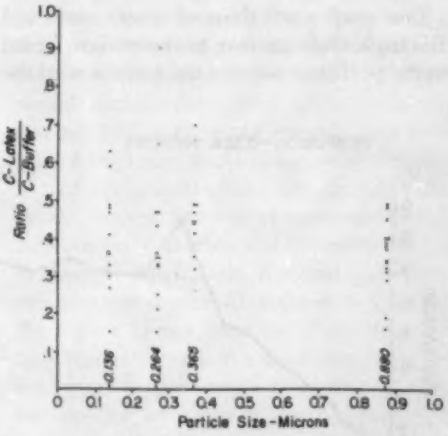

Fig. 3 (Peter, Lyda, and Krishna). Scatter ratio $\frac{\text{C-Latex}}{\text{C-Buffer}}$ per particle diameter.

Particles of latex were found embedded in the trabecular meshwork in what appeared to be a solid mass. In most sections, particles could be seen sparsely scattered in Schlemm's canal. In some of the sections of each particle size a few particles could be found in both the scleral and episcleral vessels. These findings would seem to indicate that at least some particles of each size would pass through Schlemm's canal and eventually into the episcleral plexus.

The relatively few particles that did pass through the episcleral plexus were of insufficient number to produce any blockage of the vascular channels. The blockage that did occur was confined to the trabecular meshwork. From the histiologic studies at least, there was no indication of any difference between the various particle sizes as far as penetration into the episclera was concerned. Any blocking effect the mass of particles produced was in the anterior chamber and the trabecular meshwork. However, the histiologic studies confirmed the findings of many authors that direct communications exist between the trabeculum and Schlemm's canal.

Perfusion with hyaluronidase

Our studies and those of others confirmed Bárány's findings that hyaluronidase added to the perfusate reduces the resistance of the chamber angle by approximately 50 percent. An alteration of hyaluronidase-sensitive mucopolysaccharides within the trabeculum has been suggested by Bárány as the reason the resistance is decreased. If there is a modification of the size of the intratrabecular spaces, at the same time it could be hypothecated that such a change in pore size may be reflected upon the resistance of latex particles of controlled diameters. To investigate this possibility the perfusion technique was modified slightly in that after the latex particles of a known size were perfused into the anterior chamber of enucleated rabbit eyes and a steady state reached, buffered hyaluronidase* solution from the second pipette entered the anterior chamber. With the hyaluronidase in the perfusate there was essentially no change in the resistance (fig. 4). In the majority of eyes the steady-state continued without alteration. In approximately 20 percent of the eyes the initial inflow of hyaluronidase was accompanied by a slight decrease in the resistance in the range of 12 to 18 percent. In no case was the change in resistance greater than 20 percent. This effect was expended following 0.02 or 0.03 ml. of further perfusion. Varying the strength of the hyaluronidase from 15 to 75 turbidity re-

* Testicular hyaluronidase supplied by Wyeth Laboratories, Inc., Philadelphia.

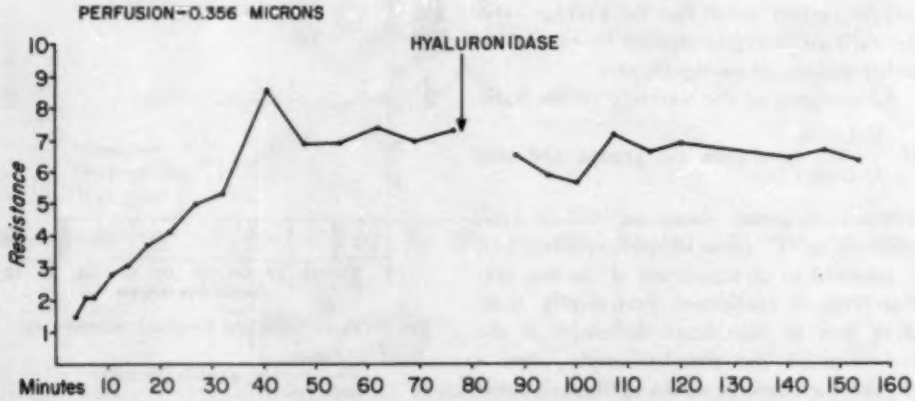

Fig. 4 (Peter, Lyda, and Kirshna). Tracing of perfusion. Latex particle with added hyaluronidase.

ducing units per milliliter did not modify the results.

It would appear that hyaluronidase produced no change in pore size of the latex occluded chamber angle as measured by change in resistance. These findings were similar to Huggert's in which he found no change in the composition of the filtrate between normal and hyaluronidase treated eyes. François et al. felt there was an enlargement of the trabecular clefts and the orifices into Schlemm's canal following hyaluronidase.

Discussion

Previous studies utilizing bacteria suffer from several inherent variables. The size of bacteria vary within the strain and also during the same stage of their growth cycle. Chemical particles such as chromium phosphate are rather heterogenous in size within limits and frequently aggregate into units much larger than their original size. It is extremely difficult to ascertain if the aggregate occurred in the anterior chamber or after perfusion. It was hoped that monodispersed latex particles would eliminate these objections. The latexes provide a series of test solutions, each limited to polystyrene particles of exactly the same size and solutions in which the exact concentration of particles can be controlled. In addition, the particles are inert and manifest essentially no tendency to aggregate or change in size.

Studies by Theobald, Ashton, and others indicated that there were open pores between the trabecular spaces and Schlemm's canal. We found that latex particles of sizes 0.13 to 1.11 μ pass through the trabecula as microscopic studies revealed the particles of each size in Schlemm's canal and the episcleral plexus.

We purposely used only those particles that would go through the trabecula to see if blockage of the spaces by particles would indicate any difference physiologically in the sizes of the trabecular spaces.

If the trabecular pores are heterogenous in size, perfusion with the smaller particles would have less resistance for the larger openings are not blocked completely by small particles, whereas perfusion with the larger particles would have a greater resistance for a larger number of the pores would be occluded. The correlation between particle size and the change in resistance would indicate a distribution of pore size within the angle of the eye. Statistical analysis reveals the resistance to be essentially the same for all particles studied.

Study of the microscopic material and the tracings revealed why we should find essentially equal resistance values with different sized latex particles. Microscopic studies indicated that though latex of each size pass through the trabecula, most of the particles are banked in and against the trabecula. That the anterior chamber was eventually filled with particles would seem to indicate that the preponderance of particles created the resistance. That it was actually the particles within the trabecula is shown by the sudden levelling off of resistance when the angle was macroscopically blocked. The resistance remained stabilized on continued perfusion even after filling the anterior chamber with particles.

Though openings do exist between the intratrabecular spaces and the inner wall of Schlemm's canal, the number of these openings would appear to be limited. Thus, it would appear that latex particles become trapped within the trabecula not as a function of the trabecular size per se or the number of trabecular spaces per se but rather by the limited number of openings present between the trabecula and the inner wall of Schlemm's canal. Thus, it would appear that the greatest degree of resistance occurs in the region of the junction of the trabecula to Schlemm's canal. We have considered this analogous to open-angle glaucoma in which the smaller openings in the trabecula and inner wall of Schlemm's canal might be blocked by hyalinization.

In no eye did the latex particles produce a complete mechanical obstruction. That the flow continues even after blockage of the

angle would indicate that some particles may continue to pass through the outflow channels but rather we feel that the greatest portion of the flow is fluid passing between the interfaces of the latex particles much as water filters through gravel. Previous studies,* in which the outflow by weight was measured, failed to recover from three to eight percent of the inflow. It is assumed that this fluid may infiltrate back into the ciliary body and suprachoroidal space and that some fluid is expended in scleral distention.

We can conclude that increased resistance to outflow with the particles smaller than the trabecular spaces and presumably larger, increases resistance by blocking the trabecular meshwork and probably at the level of the inner wall of Schlemm's canal. The increase in resistance that takes place in the trabecula is not influenced greatly by hyaluronidase.

Summary

1. The change in resistance in the chamber angle of enucleated rabbit eyes was studied by perfusing latex particles of 0.13, 0.26, 0.36, and 0.88 μ diameter.

2. No significant correlation between the diameter of the particle utilized and the change in the resistance could be found.

3. Addition of hyaluronidase to the perfusate produced no change in resistance in latex-treated eyes.

4. The intracameral perfusion of enucleated rabbit eyes with latex particles of less than one-μ diameter and their microscopic study revealed the particles to be found in the trabecula as well as in the scleral and episcleral vessels.

5. It would appear that the greatest degree of resistance to latex perfusion occurs in the region of the inner wall of Schlemm's canal.

1528 Medical-Dental Building (1).

* Unpublished report.

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Discussion

Dr. Peter C. Kronfeld (Chicago): This very interesting study by Dr. Peter and his group establishes the fact that latex particles in sizes smaller than the narrowest portion of the outflow channels are capable of impeding the flow through the corneal trabeculas of the rabbit, if such particles are added in five-percent concentration to a buffered saline perfusion fluid.

Microscopic examination of frozen sections shows these particles to form solid masses in the intertrabecular spaces. The essayists' interpretation that the preponderance of those particles in the trabecular area cause the increased resistance seems very plausible to me.

In a system of passageways as winding, intricate, and full of blind alleys as the intertrabecular spaces, it is conceivable that massing and accumulation of particles occurs, particles which by themselves, that is, individually, could very easily move forward or backward.

Dr. Peter's study, I believe, is a beautiful confirmation of some work done by the late Dr. Jonas S. Friedenwald, in 1932, when he blocked the outflow channels of living animals with homologous serum. He did this work for the purpose of estimating the rate of aqueous flow in living animals, and by an ingenious method he gradually replaced the aqueous with serum of the same animal.

There is a very striking parallelism between the two sets of experiments by Dr. Peter and Dr. Friendenwald. The concentration of colloidal particles that were injected was about the same. The particles used by Dr. Friendenwald were not of uniform size, but on the whole they were smaller than the latex particles used by Dr. Peter.

The results of the two sets of experiments were strikingly similar. The increase in resistance to flow was almost the same in the two sets of experiments.

The point brought out by Dr. Peter is that interaction of particles, which by themselves would not have the quality of blocking the angle, can affect aqueous outflow by mass action, and I think that fact should be a lead for future research.

There is at least the possibility that the anatomic changes in the trabecular area of cases affected with simple glaucoma, which we now know quite well, are not a primary degeneration but are perhaps secondary to some flow disturbance brought on by a still unknown and not really investigated anomaly of the chemistry of the aqueous.

Dr. Peter has concerned himself primarily with the immediate effects of injections of particles of a certain size into the anterior chamber. The late effects are also interesting.

Dr. Huggert, of Sweden, just made another contribution by giving single or repeated injections of particulate matter and then observing the ocular tension in living animals for periods of several weeks. Under the conditions of these experiments, there was a relationship between particle size and elevation of tension. Unfortunately, Dr. Huggert did not avail himself of the advantage of tonography, which would have enabled him to distinguish between effects on the outflow channels and masking of the effects by the depression in the aqueous flow rate. Repeated injections in the anterior chamber are bound to affect the aqueous flow rate.

As far as I can see, this is the third era in the history of ophthalmology during which the effective pore size of the outflow channels has been very much in the limelight, and the present period is particularly fruitful.

Dr. Peter's presentation here today is a clear-cut example of that. I think we are also very fortunate in having with us at this meeting one of the key investigators of this problem, Dr. François of Belgium.

Dr. J. François (Ghent, Belgium): In a microradiographic study of human eyes we have shown that particles of more than 2.25 micra cannot be visualized in Schlemm's canal even after hyaluronidase, because the great majority of them do not pass through the pores.

Another factor, which has as much importance as the size of the pores, is the depth of the anterior chamber. We did a series of experiments involving systematic modification of the depth of the anterior chamber by reducing the volume of the posterior half of the eyeball. It was always found that the resistance showed a marked increase to the extent to which the anterior chamber diminished in width. The phenomenon was always reversible in both directions.

Dr. Norman Ashton (London, England): It occurred to me on my way here that these investigators might like to see some slides showing the anatomy of the trabecular meshwork and trabecular pores, especially since it may have some bearing on the interpretation of their work. The first slide, a flat preparation stained with methylene blue and examined by dark ground illumination, shows very clearly the striking variability in the size of the pores in the trabeculae, and the tendency for each pore to be covered on either side by the adjacent lamellae. In anteroposterior sections this overlapping arrangement is well seen.

It would appear, therefore, that the degree of porosity of the meshwork is dependent not only upon the pore size but also upon the width of the intratrabecular spaces, which necessarily varies with the degree of compactness in the meshwork. During the presentation of this interesting paper I was wondering whether injection of Neoprene into the anterior chamber might not compress the meshwork to such a degree as to give an entirely false impression of its normal porosity. In any event, this possibility should be taken into account in assessing the significance of the findings.

I was interested to hear that hyaluronidase had no effect on the passage of Neoprene because this is in accord with a similar work carried out in my own department (Pedler, 1956). Whether these findings have any bearing on the function of the

living eye, however, is still open to question.

DR. FRANK VESEY (Toledo, Ohio): The time has come when we should realize that a glaucomatous eye is a diseased eye in a diseased body. It is not such a simple problem of local degeneration of the trabeculae. It has very little to do with it. As a matter of fact, increased intraocular pressure alone is far from the disease that we dread as glaucoma.

The tremendously high pressure, uncontrollable in secondary glaucoma of the vascular diseases of occiusion of the central vein of the retina, of hypertensive, hemorrhagic glaucoma, are not the cause of this Schlemm's canal occlusion, and the Schnabel lacunar atrophy of the optic nerve, which is mislabeled as glaucoma without pressure, is the disease that blinds, and it is not due to degeneration of the trabeculae.

I myself tried to contribute in a little way to the solving of this controversy between the physiopathologic concepts and the mechanical concepts. I did experiments in which I believe I succeeded in obstructing the anterior chamber angle of rabbits without ever having been able so much as just for a minute to raise the intraocular pressure of rabbit eyes.

I have sewn up the root of the eyelids all around the circumference of the cornea of the rabbit, and never saw any increase in intraocular pressure. I have installed polyethylene tubing in the chamber angle of rabbits, with good results, with never as much as five minutes of elevation of intraocular pressure in rabbits.

Therefore, I point out to you that this is not a simple problem of occlusion of the trabeculae. I do believe that Schlemm's canal has little to do even with intraocular pressure. It may be an entirely different organ with an entirely different role.

I have seen Dr. François' wonderful microslides. Of course he labeled it as going into Schlemm's canal, but I also have seen that those particles went into the ciliary body much more readily than into Schlemm's canal.

DR. PHILIP A. PETER (closing). I wish to express my sincere thanks to Doctor Kronfeld for his instructive and helpful discussion and to the guests for their interesting comments. It is indeed an honor to hear such distinguished ophthalmologists.

It was our desire to bring to your attention the availability of the mono-dispersed latex particles. We feel the unusual characteristics of the particles distinguish them as an investigative tool through which further research by other investigators may yield more information concerning the anatomy of the normal and pathologic angle of the eye.

We are continuing this work. Currently, only limited quantities of the latex particles are inserted into the anterior chamber in the enucleated eye and similar studies are under investigation in the living eye.

A STUDY OF THE INNERVATION OF THE CHAMBER ANGLE*

PART II: THE ORIGIN OF TRABECULAR AXONS REVEALED BY DEGENERATION EXPERIMENTS

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In a previous publication[1] we reported the results of a neurohistologic examination of the trabecular meshwork in four species of vertebrates. The investigation indicated striking similarities in the innervation of this region among these species. The pattern of innervation in the chamber angle, and specifically in the trabecular meshwork, was observed to be that of a plexiform arrangement of delicate preterminal and terminal axons arising from the corneal and iridociliary plexuses. The nerve fibers appeared to end solely as free filaments; no specialized receptors or neural end-organs were noted in the anterior segments of the eyes. The histologic techniques included intravital methylene blue staining of whole mounts and silver impregnation methods on serially sectioned material.

With reference to the question of a possible nervous influence on the regulation of the intraocular pressure, it is of interest and importance to determine the origin of the axons represented in the trabecular mesh-

* From the Ophthalmology Branch, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare.

work in view of the role this region plays in the aqueous outflow mechanism. Accordingly in the present investigation a series of denervation procedures were carried out in an attempt to remove the postganglionic nerve fibers of the parasympathetic, sympathetic, and trigeminal nerve supply to the eye. The trabecular meshwork was then examined for the presence of degenerating axons.

Material and Methods

The following denervation procedures were carried out on albino rabbits weighing from one to five kilograms: opticociliary neurotomy (five animals), superior cervical ganglionectomy (six animals), ciliary ganglionectomy (seven animals), and trigeminal neurotomy (six animals). More animals were operated on, but about half of them were excluded because of failure of the technique or early incidental death.

The neurohistologic techniques comprised methylene blue vital staining utilizing whole mounts of the anterior segment and Bodian and Gross-Schultze silver impregnation procedures as outlined in the previous study. In each of the experimental animals the opposite, unoperated eye served as control and was treated identically to the eye on the operated side. In all surgical procedures anesthesia was induced by intravenously injected sodium pentobarbital in a dose of 25 mg. per kg. supplemented by ethyl ether inhalation. The postoperative care included daily injections of penicillin-streptomycin mixture (Combiotic, 2.0 cc.) for the first postoperative week. In the animals subjected to trigeminal neurotomy, the lids of the operated side were kept closed by sutures to prevent the development of a neuroparalytic keratopathy. The animals were killed at appropriate time intervals by an overdose of intravenously injected sodium pentobarbital. The operative techniques used in the denervation experiments are outlined as follows:

1. Opticociliary neurotomy

All nerves entering the eye were severed at the posterior pole in order to establish the histologic appearance of degenerating axons in the area of interest. The technique was similar to the procedure used clinically on the human eye.[3] The posterior pole of the globe was exposed by an approach from the temporal side after the lateral rectus was detached. The optic nerve, long and short ciliary nerves, and the vessels were then severed with curved enucleation scissors. The region of the posterior pole of the eye was then cleaned of fascia, nerves, and vessels, and the entire field cauterized with a thermal cautery kept to as low an effective temperature as possible. After attachment of the lateral rectus the wound was closed.

2. Superior cervical ganglionectomy

Since the operative procedure is well known,[3] no details are given here. No animal was included in the series unless removal of the ganglion was verified histologically. The clinical signs of the ganglionectomy were considered ancillary.

3. Ciliary ganglionectomy

The procedure in this study resembled that described by Shen and Cannon[4] but was modified by a purely intraorbital approach; no bone, fascia, or muscles were removed as in the extraorbital method. The motor nerve to the inferior oblique was identified at the point where it passes below the medial margin of the inferior rectus and is imbedded in the fascia of the Hardarian gland. The course of this nerve guided the blunt dissection of a fascial plane leading deep into the orbit to the junction of the branch with the oculomotor stem. A slender retractor was then inserted along the nerve to keep fascia and muscles out of the operation field.

The operative procedure was aided by the use of the Zeiss otoscope. The $\times 10$ magnification greatly faciltated the identification of the site of the ganglion, since in the rabbit it

rarely exceeds 0,4 mm. in its greatest dimension.[5] Just above the point of junction of the motor nerve to the inferior oblique with the oculomotor stem the ganglion can be visualized. It lies deeply in the apex of the orbit lateral to the optic nerve and medial to the external rectus muscle. It sometimes forms a nodular swelling on the medial surface of the third nerve, and its short ciliary branches appear as though they were the first division of the oculomotor stem.

The ganglion was excised with a short piece of the oculomotor nerve to which it is attached. The short ciliary nerves were severed close to the ganglion to complete the excision. By taking the precaution of sectioning these nerves close to the ganglion and by keeping in the immediate vicinity of the oculomotor stem it was felt that the excision of the ganglion could be accomplished without disturbing the long ciliary nerves.

The animals showed fixed, dilated pupils on the operated side, but none were included for evaluation unless there was histologic proof of complete removal of the ganglion.

4. Trigeminal neurotomy

The operative procedure followed the standard extradural approach in the middle cranial fossa as described for primates.[6] After the temporalis muscle was severed from its origin and deflected, a 1.5 cm. hole was trephined in the temporal bone as far inferiorly as possible, just above the temporo-mandibular joint. This opening was enlarged with ronguers to about twice its diameter. The dura was bluntly separated from the bone by wedging Cottonoid between the two structures; this packing procedure also controlled bleeding.

By gentle retraction of the brain the postganglionic branches of the fifth nerve could be seen under ×10 magnification, although sometimes with difficulty. They run in a groove covered by dura. After the dura was deflected the nerve branches were cut with a thin bladed knife or interrupted by avulsion.

It was necessary to extend the section of the nerve deeply into the floor of the groove and as far medially as possible because the ophthalmic branch is situated in the innermost part of the groove and often slips under the blade or forceps in the process of severing the nerve. In the closure procedure the bony defect was filled supradurally with gelfoam. In some animals the temporalis muscle was reattached.

A troublesome postoperative complication consisted of asymmetric closure of the mouth, which prevented the animals from chewing. Under this condition it was difficult to keep the rabbits alive beyond five or six weeks. Suturing of the lids as a rule prevented the formation of keratitis; in one animal only the cornea became slightly cloudy in one quadrant. The criteria for successful trigeminal neurotomy were clinical. The degree of anesthesia of the lids was tested by crushing the skin with a hemostat and of the cornea by touch with a cotton wisp. The absence of the reaction to skin trauma or of the blink reflex upon corneal touch was compared with the responses of the eye on the unoperated side.

Neurotomy was considered complete on clinical grounds in three animals of this series. The remaining three rabbits suitable for study were adjudged incomplete neurotomies. These animals exhibited decreased or absent sensation in some parts of the lids, but sensation appeared to be intact in other portions. The corneas of all these animals displayed diminished or absent sensation; however, a blink reflex could be elicited in some areas or could be evoked by summation; that is, by dragging the cotton wisp over a small area of the cornea. In two of the animals the site of section was determined by post mortem and histologic examinations, which ascertained that all postganglionic fibers were cut.

The present report contains also a brief description of the innervation of the human trabecular meshwork of an eye, the normal

anterior segment of which was stained intravitally with methylene blue and mounted as a whole.

Observations

The observations are grouped according to the following scheme:

A. The innervation of the human trabecular meshwork.

B. Axonal morphology in the rabbit trabecular meshwork following:

1. Opticociliary neurotomy
2. Ciliary ganglionectomy
3. Superior Cervical ganglionectomy
4. Trigeminal neurotomy

A. Innervation of human trabecular meshwork

The results of the methylene blue method applied to the human trabecular meshwork corroborated previously reported observations which were based on serially sectioned silver-stained material. In addition the whole mount technique permitted a comprehensive view of the total innervation of the meshwork.

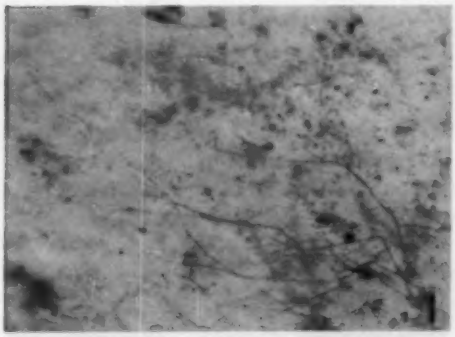


*Fig. 1 (Holland, von Sallmann, and Collins). Axonal arborization in the human trabecular meshwork (×215).

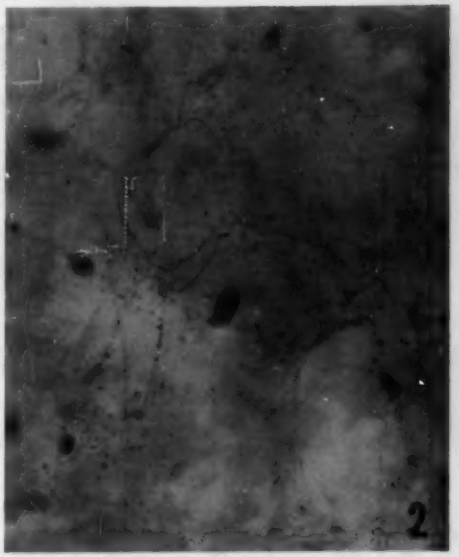


Fig. 2 (Holland, von Sallman, and Collins). Relationship of terminal axonal arborization to fibers of the trabecular meshwork. The oval, clear areas represent the spaces between individual, semitransparent trabecular bundles. A preterminal axon loops among several trabeculae, gives off one or two fine branches, and finally runs along the margin of a trabecula to end near its base as a free filament (×430).

The preparation revealed a striking abundance of nerve fibers in this region, a fact which was not fully appreciated in the silver stained sections. An immense number of nerve fibers extended from the ciliary and iridociliary plexuses to arborize in many different planes in the trabecular meshwork (fig. 1). In general, bundles seen in the human eye were less conspicuous than those seen in the rabbit, since the former were recognized only with magnification, whereas they could be distinguished grossly in a methylene blue vitally stained preparation

* All of the illustrations herein are from methylene blue vitally stained material *except* Figures 18 and 21, which are from sections stained by silver methods of Gros, Schultze and Bodian, respectively.

Figures 1 and 2—human eye.

Figures 3 through 25—rabbit eye.

Figure 3—opticociliary neurotomy.

Figures 4 through 13—ciliary ganglionectomy.

Figure 14 through 19—superior cervical ganglionectomy.

Figures 20 through 25—trigeminal neurotomy.

The magnification and the times of death are indicated in each figure.

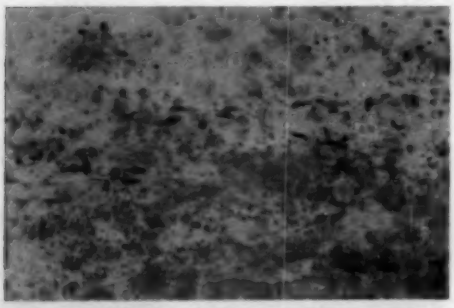


Fig. 3 (Holland, von Sallmann, and Collins). Absence of nerve fibers in trabecular meshwork following total denervation. A few scattered Schwann elements remain (2.5 wk., ×223).

of the anterior segment of a rabbit eye. Aside from this difference the innervation of this region was very similar in the two species.

Trabecular nerve fibers which originate from corneal nerves were also present in the human. The type of ending noted was that of the free axonal filament; no specialization of endings was evident. By using the whole mount it was also possible to study the topographic relationship between the axonal arborizations and the trabeculae. In Figure 2 the spaces in the semitransparent trabecular network can be visualized.*

A preterminal axon runs on and among several trabecular lamellae in a looped course in slightly different planes. The preterminal axon gives off one or two fine branches and finally follows the margin of one trabecular fiber to end as a free axonal filament near the base of this fiber. There seems to be no regular arrangement of nerve fibers with respect to the architecture of the trabecular meshwork; they appear to be distributed at random. The arborization of axons was observed in all parts and layers of the meshwork but none were seen beyond Schwalbe's line where the trabecular fibers insert.

* When ideal staining has occurred only nerve fibers stain with the dye, the other tissue element remaining clear.

B. AXONAL MORPHOLOGY IN RABBIT TRABECULAR MESHWORK FOLLOWING DENERVATION EXPERIMENTS

1. *Opticociliary neurotomy.* The eyes of five animals were subjected to this procedure. Three of these eyes were examined after methylene blue vital staining (two killed one week postoperation, and one at two and one-half weeks) and two were examined utilizing the Bodian and Nauta[7] silver techniques (both killed at one and one-half weeks).

After total denervation axons in the trabecular meshwork as well as those in the iris and cornea were few in number and those observed were in various stages of degeneration at one week. Occasionally seen were isolated fragments of completely disorganized axons or vestiges of Schwann sheaths, but most conspicuous was a marked diminution or complete absence of all nerve fibers in the trabecular meshwork (fig. 3).

One or two days after this operation the cornea usually showed edema, and neovascularization occurred along the entire limbus; the lens and cornea became opaque afterward. Due to this severe reaction to the surgery and the onset of phthisis it was not worth while to examine eyes much later than a week or two after operation.

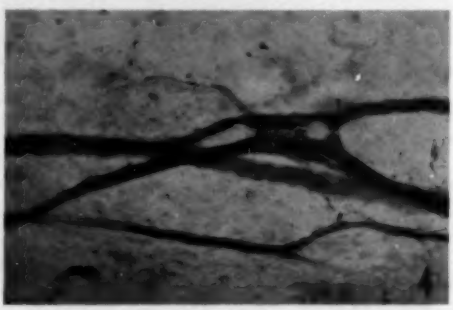


Fig. 4 (Holland, von Sallmann, and Collins). A relatively well-preserved, palely stained bundle of axons contrasting with a darker stained H-shaped configuration of more severely affected degenerating nerve bundles. The latter shows axonal fragmentation and proliferation of Schwann nuclei (4 wk., ×130).

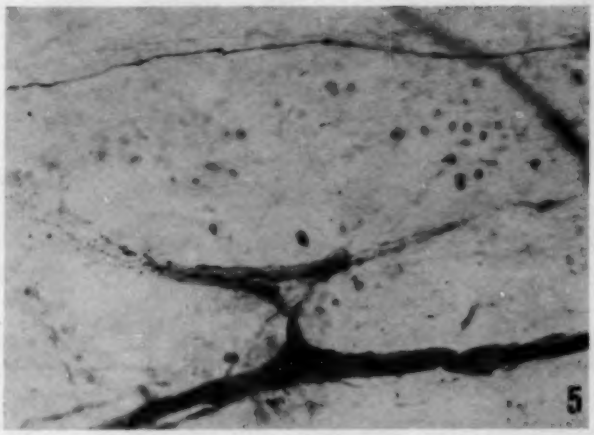


Fig. 5 (Holland, von Sallmann, and Collins). A small nerve in the upper field shows a single, thickened, degenerating axon with varicosities and fractures adjacent to thinner smooth normal fibers. The H-shaped pattern of nerves in the lower field shows prominent sheath, proliferation of Schwann cell nuclei, degenerated axon fragments in the lower limb, and a fainter sheath with attempt at regeneration (toward the left in the upper limb, see Figure 6) (4 wk., ×130).

2. *Ciliary ganglionectomy.* Methylene blue vital staining was utilized in five animals of this series (killed at intervals of 3, 4, 5, 7, and 7.5 weeks) and two were killed four weeks after surgery and the eyes reserved for silver impregnation.

In all preparations fairly extensive, unequivocal degeneration of axons was observed in the meshwork (figs. 4, 5, 6, 7, and 8). The axonal changes which occurred in the eye stained three weeks after surgery were confined to disturbances in morphology consistent with early degeneration, for example, axonal thickening, vesiculation, varicosities, fragmentation of axons, and beading without continuity, sometimes hyperstaining of individual fibers, and occasionally granular disintegration (fig. 9). In addition to these changes, the other slightly older preparations showed the presence of empty Schwann sheaths, metachromasia of sheaths and degenerating fibers, proliferation of Schwann cell nuclei, increased staining of Schwann sheaths, and complete granular disintegration of axons, often leaving portions unattached to the parent bundle. Various phases of degeneration were often present simultaneously (fig. 10), and not infrequently degenerating nerve fibers could be seen coursing in the same bundle with rela-

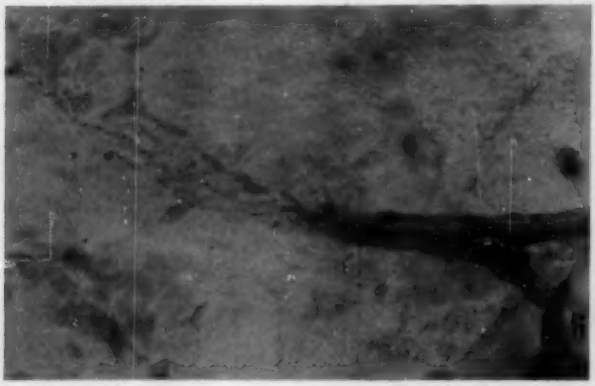


Fig. 6 (Holland, von Sallmann, and Collins). Higher-power view of part Figure 5, showing delicate, finely beaded axons indicating regeneration (×800).

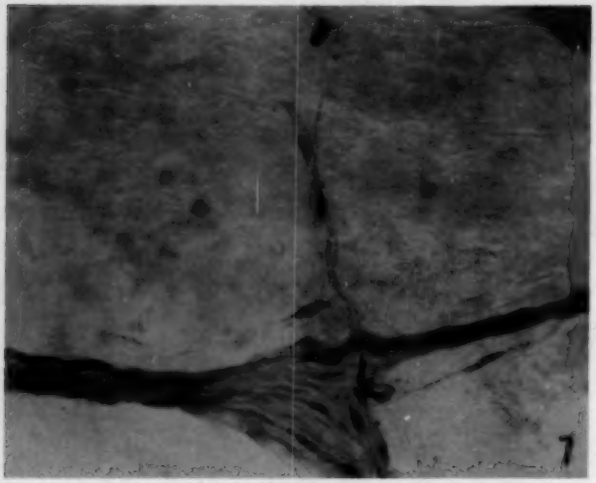


Fig. 7 (Holland, von Sallmann, and Collins). The nerve bundle shows staining of the sheath, proliferation of Schwann nuclei, a prominent degenerating fragmented axon, and several relatively better preserved nerve fibers. The vertical branch consists of Schwann nuclei interconnected by fibrillae (4 wk., ×750).

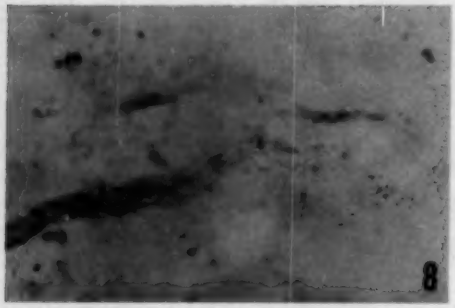
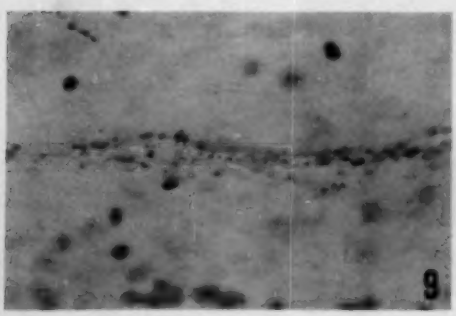


Fig. 8 (Holland, von Sallmann, and Collins). Degeneration and regeneration of axons in the trabecular meshwork proceeding concurrently after ciliary ganglionectomy. The lower portion of the nerve shows no axons, only a stained sheath with nuclei, while the upper portion shows a clear, fine, regenerating fiber, emerging from the nerve sheath (7 wk., ×210).



tively well-preserved axons (fig. 11).

There was perhaps some slight decrease in the over-all number of small nerve fibers when compared with control eyes; however, this difference was not impressive when compared with the striking absence of axons following total denervation. In other words, in spite of fairly extensive morphologic evidence of nerve fiber degeneration, marked absence of axons was not a characteristic feature.

Scattered fibers were observed which had the morphologic characteristics ascribed to regenerating axons (figs. 12 and 13). They could be recognized as very thin, straight, irregularly beaded fibers, sometimes branching at right angles from a bundle of axons showing degenerating fibers, and rarely terminating in growth cones. The eyes examined four weeks after surgery appeared to be optimal for the detection of degeneration and regeneration.

Fig. 9 (Holland, von Sallmann, and Collins). The degenerating axons in the bundle have broken up into discontinuous beadlike fragments (3 wk., ×465).

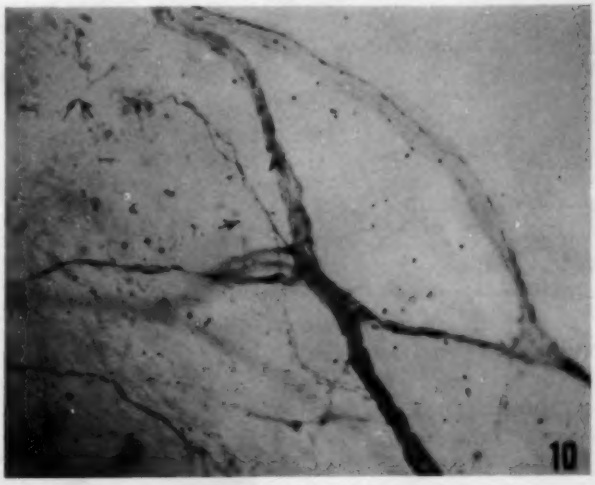


Fig. 10 (Holland, von Sallmann, and Collins). Over-all view of the innervation of the trabecular region. The simultaneous presence of degenerating, regenerating, and normal nerve fibers. The large X-shaped bundle contains normal and degenerating axons, staining of the sheath, as well as prominence of Schwann cell nuclei. The group of axons in the lower left corner shows fragmentation, and the connecting bundle in the upper right field shows a number of normal fibers and one axon coursing in the center which clearly shows degenerative changes. The central arrow indicates very thin regenerating fibers (see fig. 12) and the upper left arrow points to an empty Schwann tube and Schwann nuclei (4 wk., ×130).

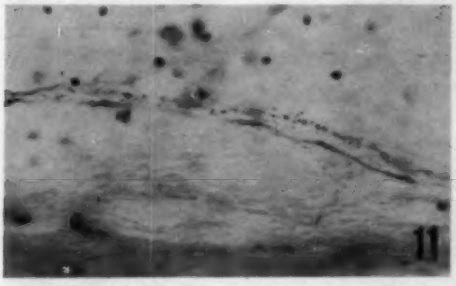


Fig. 11 (Holland, von Sallmann, and Collins). A relatively normal axon adjacent to one showing degeneration in the form of granular disintegration and discontinuous beading (3 wk., ×235).

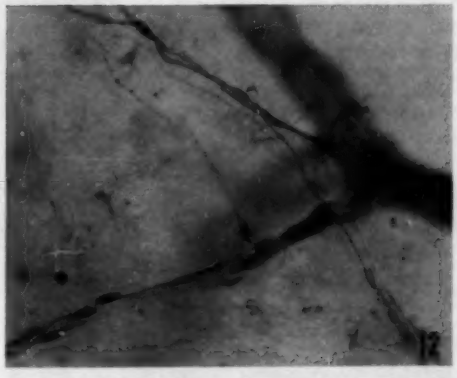


Fig. 12 (Holland, von Sallmann, and Collins). Higher-power view of part of Figure 10, showing a collateral branch of fragile, beaded, regenerating axons (4 wk., ×700).

3. *Superior cervical ganglionectomy.* Five rabbit eyes were examined after methylene blue vital staining (killed at intervals of 2.5, 5.75, 6, 10, and 13 weeks), and one animal was killed four weeks after surgery and the eyes stained by the silver methods.

Axons in various stages of degeneration were observed in the meshwork in every preparation (figs. 14, 15, 16, 17, and 18).

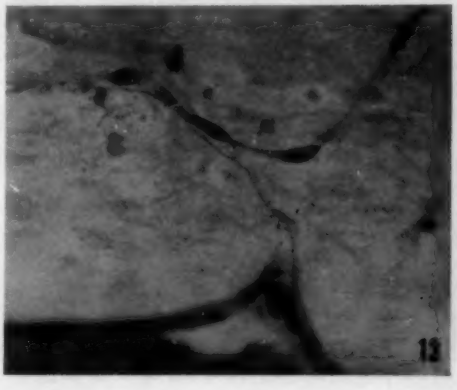


Fig. 13 (Holland, von Sallmann, and Collins). A very thin, finely beaded, regenerating axon emerging from the large bundle of degenerating nerves below, and coursing along the Schwann sheath above (4 wk., ×1,100).

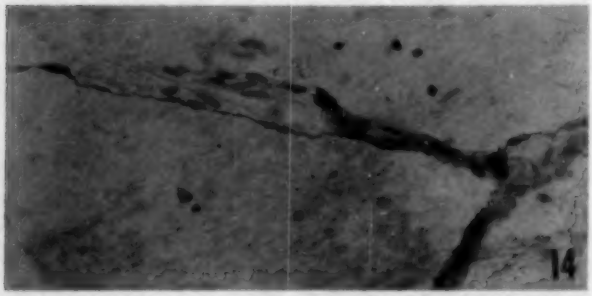


Fig. 14 (Holland, von Sallmann, and Collins). Degenerating nerve bundles consisting mainly of sheath and fragmented axons (5.75 wk., ×285).

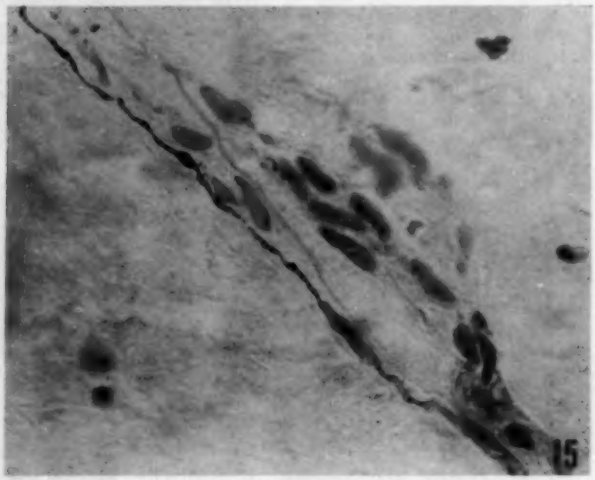


Fig. 15 (Holland, von Sallmann, and Collins). Higher-power view of part of Figure 14, showing Schwann sheath, prominent Schwann nuclei, and a thickened, varicose, fragmented axon (×1,300).

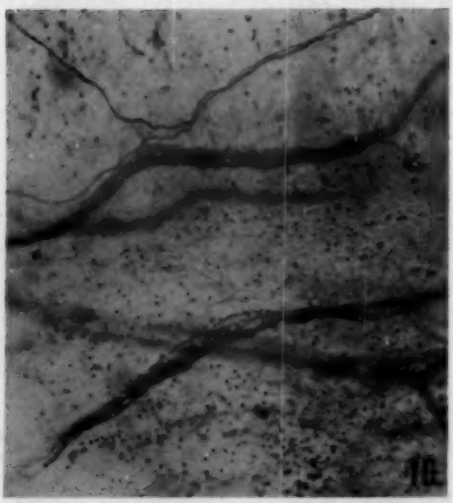


Fig. 16 (Holland, von Sallmann, and Collins). Lower-power view of trabecular area. A large almost totally degenerated nerve fiber in the center of the field; the bundle above this is less severely affected (13 wk., ×110).

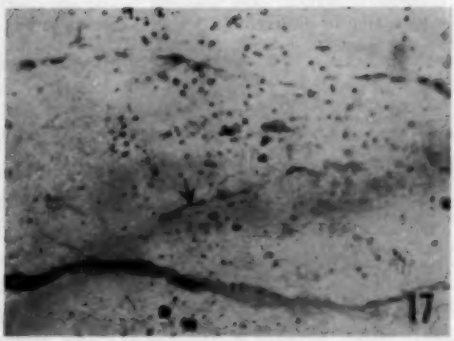


Fig. 17 (Holland, von Sallmann, and Collins). Above: an empty Schwann sheath. Below: a larger fiber, showing degenerative changes. Arrow indicates a detached axonal fragment (13 wk., ×145).

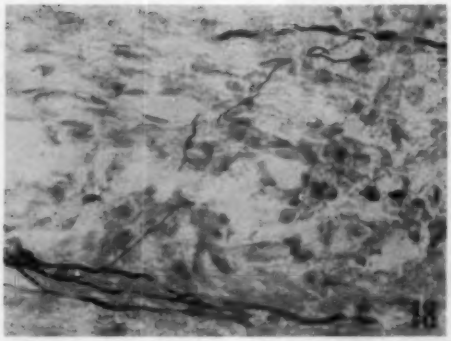


Fig. 18 (Holland, von Sallmann, and Collins). The axons in the upper right field are thickened, varicose, and fragmented (4 wk., ×385).

The morphologic characteristics of the degenerating fibers was the same as reported in the animals subjected to ciliary ganglionectomy, except that the lesions were much less extensive. The eyes examined six weeks after surgery seemed best suited for the study of degenerative changes. Although it was not difficult to demonstrate degenerating nerve fibers in the meshwork, the over-all density of innervation (including both normal and degenerating fibers) did not appear to be diminished appreciably by this procedure. Only a few regenerating axons were observed (fig. 19).

4. *Trigeminal neurotomy.* Four eyes of this group were stained vitally with methylene blue. Two of these were bisected, and one half of the eye reserved for silver impregnation, thus affording the opportunity of comparing both techniques on the same eye. A supravital methylene blue stain was done on one of the eyes, and the eyes of another animal were prepared for silver staining. The rabbits were killed: two at two and one-half, two at four, one at five, and one at six weeks postoperatively.

After severing the postganglionic nerve fibers of the fifth nerve intracranially, examination of the trabecular meshwork revealed axons in various stages of degeneration (figs. 20, 21, and 22). In the completely neurotomized animals all of the corneal nerves, as well as many axons in the trabecular meshwork and iris, were seen to be in some phase of degeneration. The incompletely neurotomized animals tended to show fewer degenerating fibers. Despite the presence of such fibers, the over-all density of innervation of the meshwork did not appear to be definitely reduced. Scattered regenerating fibers were also noted (figs. 23 and 24), and often degenerating branches from corneal nerves could be traced to the trabecular

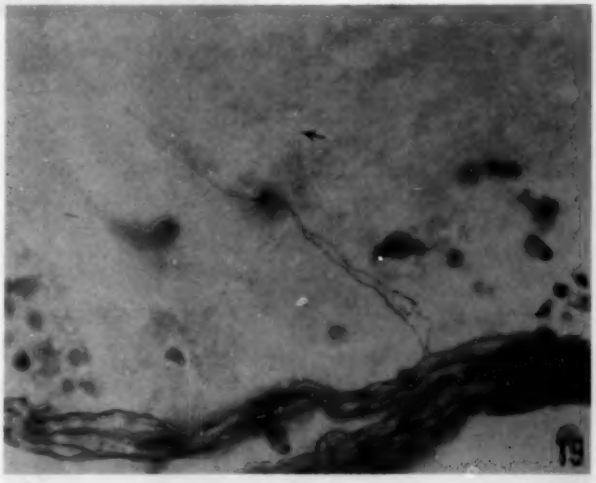


Fig. 19 (Holland, von Sallmann, and Collins). A pair of regenerating axons arising from a large bundle containing thickened, vesiculated, fractured fibers. Arrow indicates a delicate, thin, beaded, regenerating collateral branch (6 wk., ×1,120).

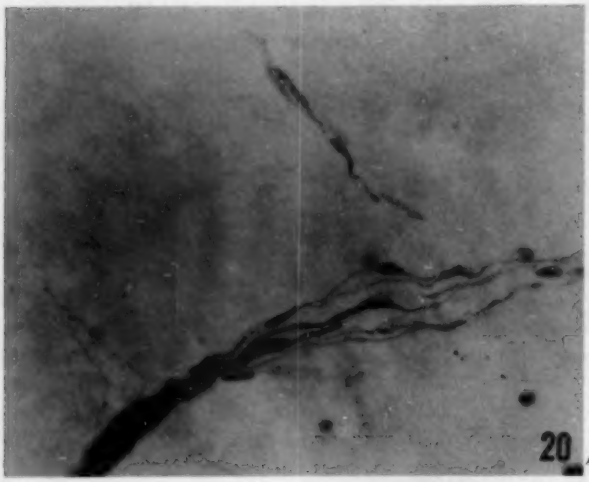


Fig. 20 (Holland, von Sallmann, and Collins). A degenerating nerve bundle with stained sheath, prominent Schwann cell nuclei, and axonal thickening and fragmentation. An adjacent isolated Schwann sheath and nuclei in the upper right field, and delicate, beaded, regenerating fibers in the lower left field, coursing from the large bundle (4 wk., ×417).

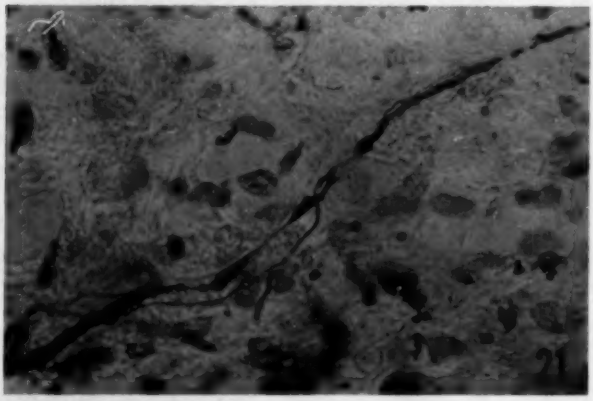


Fig. 21 (Holland, von Sallmann, and Collins). Axonal thickening, fractures, and varicosities alongside relatively well-preserved fibers (2.5 wk., ×1,200).

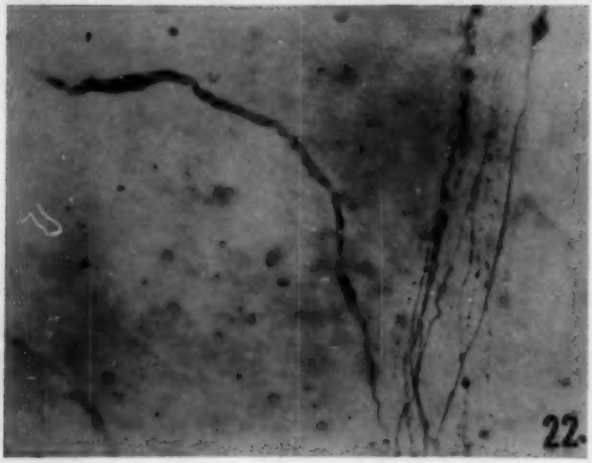


Fig. 22 (Holland, von Sallmann, and Collins). Large nerve bundle containing axons in several stages of degeneration, which gives a collateral branch to the trabecular area before entering the cornea. The degenerating trabecular branch shows axonal thickening, varicosities, and fractures (4 wk., ×235).

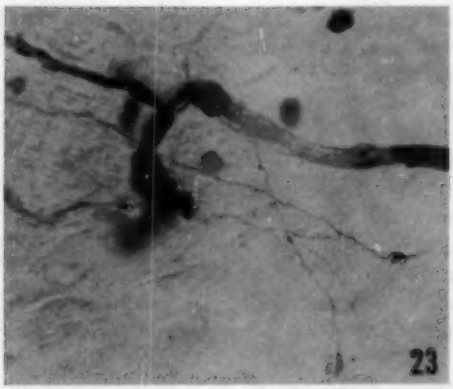


Fig. 23 (Holland, von Sallmann, and Collins). A higher-power view of a portion of Figure 25. Several delicate regenerating axons near a degenerating fiber, the latter consisting mainly of a sheath and axonal fragments. Note the convolution of the regenerating fiber on the right side of the field (5 wk., ×1,650).

region (fig. 25). The characteristics of the degenerating and regenerating fibers were the same as mentioned in the other experimental groups. In general, the eyes examined four to six weeks following trigeminal neurotomy appeared optimal for detecting degeneration.

Discussion

The evidence reported in this study strongly suggests that the axons represented

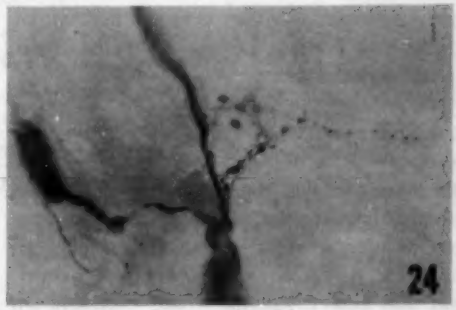


Fig. 24 (Holland, von Sallmann, and Collins). Degenerating axons showing fragmentation and thickening alongside a delicate, finely beaded, regenerating fiber (4 wk., ×1,380).

in the trabecular meshwork come from at least three sources, namely, the sympathetic (via the superior cervical ganglion), and parasympathetic (via the ciliary ganglion) divisions of the autonomic nervous system, and from the fifth cranial nerve. Before attempting an estimate of the relative proportion of fibers contributed by these components, it is first necesary to consider some of the great limitations involved.

The material studied did not lend itself to the quantitation of the degenerating axons present because of the complicated arborization of the nerve fibers in various planes of the trabecular meshwork. Even a rough appraisal of the relative proportion of the vari-

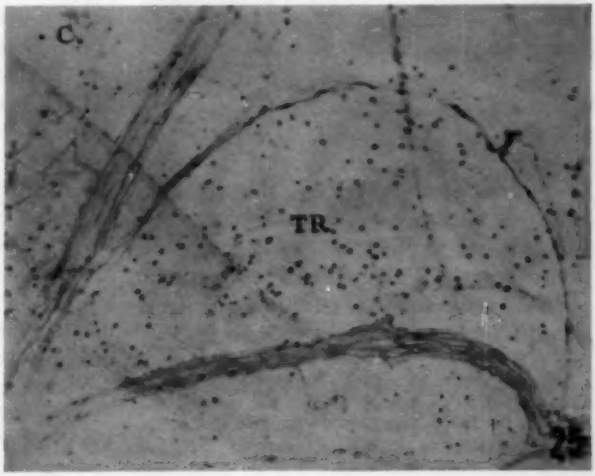


Fig. 25 (Holland, von Sallmann, and Collins). A degenerating trabecular branch from a nerve supplying the cornea. The latter shows thickened, fractured, and varicose axons. Higher-power view of degenerating trabecular branch in Figure 23. Also shows regenerating fibers (5 wk., ×143). (C) Cornea. (Tr.) Trabecular region.

ous participating fibers presents substantial difficulties because of the following additional considerations.

It is not possible to section all postganglionic axons of sympathetic and parasympathetic ganglia which supply nerve fibers to the eye. It is well known, for example, that accessory sympathetic[8] and parasympathetic ganglia[8] exist in the rabbit. In this case, then, excision of the superior cervical and ciliary ganglia would represent an incomplete removal of the respective postganglionic nerve fibers. Nonetheless, extirpation of these two ganglia probably will irradicate the larger part of the autonomic nerve fibers to the eye.

The ciliary ganglion in the rabbit may lack a sympathetic root[8] but there is fairly good agreement in the literature that the long ciliary nerves send fine branches through the ganglion. Thus, excision of the ganglion is accompanied by sectioning of some fifth nerve fibers; however, since the number of these fibers is relatively small, this does not constitute a serious objection, and the ganglionectomy can be considered as essentially interrupting parasympathetic pathways.

Similiarly, sympathetic fibers to the eye course in the nasociliary branches of the trigeminal nerve, so that sectioning of the fifth nerve may not exclusively remove somatic fibers. However, the trigeminal neurotomy was located just distal to the gasserian ganglion, as corroborated by post mortem and histologic examination, whereas the sympathetic fibers to the eye (in primates[9, 10]) join the ophthalmic branches anterior to the site of section; that is, at the level of the carotid-cavernous sinus plexus. It has not been shown clearly whether or not the pertinent anatomic details described in primates apply to the rabbit.[3, 11]* Thus, the possibility cannot be excluded that some sympathetic fibers associate with the gasserian ganglion or enter the fifth nerve proximal to it. Under these conditions the fibers would be severed at the site of the neurotomy.

Another complicating factor concerns the phenomenon of collateral regeneration. Briefly this means that in a partially denervated region regenerating axons may arise from the adjacent normal nerves and grow into the denervated area, in an attempt as it were to restore the inervation. This phenomenon has been observed to occur in both sensory and motor nerve fibers.[12–15] Hence, removing the source of one component of the regional innervation; for example, the parasympathetic, does not imply that the other component nerve fibers will remain "morphologically quiescent." Weddell[16] has shown, for example, that after sectioning some of the corneal nerves in one quadrant of the cornea, the other apparently undisturbed nerves send regenerating fibers to the denervated region. These new fibers eventually disappear as regenerating axons from the severed nerves re-enter the cornea; because of this phenomenon it might be difficult to judge the relative contribution of one component to the total innervation of an area.

It might also be mentioned at this point that the optimal uniformity of staining from preparation to preparation was difficult to achieve. This factor added to the difficulties in evaluating nerve depletion.

It is evident, then, that not more than a rough estimate can be made of the degree to which the parasympathetic, sympathetic, and fifth nerve fibers participate in the innervation of the trabecular meshwork. In comparing the results in the three groups it appears likely that many of the nerve fibers present in the meshwork are parasympathetic in origin, and that a smaller number arise from the trigeminal and sympathetic nerves. To determine whether there is a bona fide difference in the relative proportion of fibers contributed by the sympathetic and trigeminal nerves would require a larger series of

* E. S. Perkins, in a recent publication (Brit. J. Ophth. 41:257, 1957), suggests that the sympathetic fibers in the rabbit may join the ophthalmic branch as far anteriorly as the orbital fissure.

animals and different experimental approaches.

It should be emphasized that in contrast to the appearance of the angle in eyes subjected to opticociliary neurotomy, the over-all density of innervation was not strikingly diminished in the three procedures of selective denervation; many axons remained well preserved under these experimental conditions. Furthermore, even if it had been possible to carry out all three procedures on a single animal, it would seem that the combined axonal depletion would not equal that seen in a totally denervated eye.

This relative preservation of the over-all density of trabecular innervation could have been caused by a combination of several factors: It is possible that nerve fibers of other origin are represented in the meshwork, but available anatomic knowledge does not support this idea.[2, 9-11] Regeneration, especially collateral regeneration, makes it difficult to detect a diminution in nerve density; however, this phenomenon was not observed frequently enough to provide a satisfactory explanation.

It is most plausible, then, that the accessory ganglia play a greater role in the autonomic innervation of the rabbit eye than hitherto suspected. The works of Peschel[9] and Jegerow[5] amply illustrate the presence of these small, though numerous, ganglia.

To clarify the latter point it would be desirable to combine all three denervation procedures in the same animal, a difficult feat to accomplish. Also, future work dealing with the origin of trabecular axons might profitably be done on primates where the problem of accessory ganglia is minimized.

The observation that many of the trabecular axons appear to be parasympathetic in origin does not necessarily imply that they are efferent fibers. In fact, Mitchell[17] states, "Vascular and visceral structures are sensitive to adequate stimuli. Pain, pressoreceptor, chemoreceptor, and other centripetal impulses arise in the heart, vessels, and other visceral structures, yet many still adhere to the misconception of the autonomic as a purely efferent system."

Summary

1. In an attempt to ascertain the origin of trabecular nerve fibers the following series of denervation experiments were carried out on albino rabbits: opticociliary neurotomy, superior cervical ganglionectomy, ciliary ganglionectomy, and trigeminal neurotomy.

2. From the reported histologic observations, it is concluded that the nerve fibers in the trabecular meshwork are derived from at least three sources, namely, the parasympathetic, sympathetic, and fifth nerve fibers to the eye.

3. Comparison of neurohistologic observations on the totally denervated eyes with those of each of the "selectively denervated" eyes revealed the following:

a. Concurrent degeneration and regeneration of trabecular nerve fibers was consistently observed.

b. The relative proportion of degenerating nerve fibers in the meshwork following these denervation procedures can be listed in decreasing order: opticociliary neurotomy (total denervation), ciliary ganglionectomy, trigeminal neurotomy, or superior cervical ganglionectomy.

4. The neurohistology of the trabecular innervation of a human eye stained intravitally with methylene blue and examined as a whole mount is reported.

Ophthalmology Branch (14).

Acknowledgment

We gratefully acknowledge the valuable advice and constructive criticism of Dr. Darab Dastur.

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Discussion

Dr. Russell L. Carpenter: For many years we have thought we knew the structure of the iris angle, including the elements of the individual trabeculae of the meshwork and their arrangement relative to the canal of Schlemm, the scleral spur, and the ciliary body. We have had a clear picture of Schlemm's canal and the collector channels connecting it with the intrascleral venous plexus. We have long been aware also of variations in the anatomic arrangement of this region in different animal species.

The sum total of our knowledge has indicated quite clearly that this region is concerned with aqueous outflow and hence with the intraocular pressure, but increasingly the question has been raised as to whether the trabeculae of the meshwork play an active or a passive role in relation to intraocular pressure.

The first step toward an answer to this question would seem to have been the anatomic demonstration of nerve endings in the trabecular meshwork. It is usually safe to assume that structures do not exist without reason. One must assume that if the meshwork plays a role other than that of an interposed fine-meshed sieve through which the aqueous must filter to reach Schlemm's canal, then the presence of nerve terminations means that either the trabeculae act as a pressoreceptor or they function directly as a regulator of outflow, or both. The analysis of the source of these nerve terminations therefore becomes a matter of importance in understanding the functional role of this region.

I suspect we are all aware of the technical difficulties which had to be mastered to achieve the results presented here. Except in expert hands, the staining methods employed can be most capricious. Neither are the problems simple which are encountered in accomplishing selective denervation of the eye. It is therefore a matter for some slight commisseration (and puzzlement) that clear-cut results did not ensue from the clear-cut design of the experiments.

In those experiments involving complete opticociliary neurotomy, the fact that one week later only a few axons were seen in the trabeculae—and these few in stages of degeneration—is understandable in view of the general condition of the eye resulting from the severe surgical procedure. Degeneration could be expected to proceed at maximum rate.

It is difficult to understand why, in the denervation experiments which selectively eliminated axons to the bulb from the ciliary ganglion, the superior cervical ganglion, and the gasserian ganglion, respectively, the effects noted in the trabecular meshwork were not more marked. The evidence seems clear that all three contribute nerve fibers to the trabeculae. Why, then, were there not evident in the preparations either an appreciable reduction in the number of axons or else very numerous degenerating fibers?

The authors suggest several possible explanations. One is the presence in the rabbit of accessory autonomic ganglia, the postganglionic axons of which would have been spared by the experimental procedures. Accessory ganglia are not peculiar to the rabbit; in the human, ganglion cells occur along the course of the ciliary nerves, in the scleral canals, and even in the ciliary body. Despite their not infrequent occurrence, they probably

constitute in their entirety a minor portion of the parasympathetic supply.

It would be useful to know to what extent this is true of the rabbit. Serial section counts of the number of accessory ganglion cells compared to the number in the ciliary ganglion itself could be made and might be well worth-while.

The possibility of collateral regeneration having occurred extensively enough to re-innervate the area during the interval between experimental surgery and a study of the meshwork appears to be ruled out by reason of the varying intervals employed. If extensive regeneration had occurred, surely it would have been evidenced more frequently in the preparations.

Another question might be raised, namely, the extent to which in the rabbit sympathetic fibers from the cavernous plexus reach the eye by accompanying the branches of the ophthalmic artery. These would have been spared in all cases save the optico-ciliary neurotomies.

It seems clear from these experiments that an appreciable number of axons to the trabeculae course in the short ciliary nerves from the ciliary ganglion, but these may well be, as the authors have pointed out, visceral afferent fibers.

I would like to ask whether it would be possible in any of the preparations to study the trabecular endothelium for signs of trophic disturbances resulting from interruption of afferent fibers. It might also be asked whether tonometric measurements of intraocular pressure following each of the various types of denervation procedures might give some indication of what these axons in the trabeculae are doing there.

This points up, of course, the fact that it is very easy to suggest experiments for someone else to do. I am sure that we all hope these authors will further continue their studies on the innervation of the chamber angle, for their efforts so far have yielded a good harvest.

DR. MONTE G. HOLLAND (closing): Dr. Carpenter has dwelt on certain points that I could not elucidate in the brief time allotted to me. One of the points he mentioned, which is brought out in the paper, is that there appeared to be a disparity between the totally denervated eye and the eyes subjected to procedures of partial denervation in respect to the total number of nerve fibers present, that is, the over-all density of innervation.

After total denervation there was almost a complete absence of nerve fibers. After ciliary ganglionectomy there was some diminution. Following the other two procedures it was difficult to be sure that there was any appreciable change in the total nerve density.

The possibility that accessory ganglia may explain the relative preservation of nerves in this region still seems to be one which deserves further investigation.

In regard to changes in the trabecular region following denervation—we did not observe any atrophic changes in the trabecular fibers themselves. There were indications from time to time of the possible presence of reactive cells to the degenerating nerve fibers, but it was difficult to be sure of this because there were often blood cells present. However, in general there was no change noticeable in the appearance of the trabeculae themselves.

Finally, the physiologic implications of this study should be left properly to the physiologists. This is part of a broad study undertaken by Dr. von Sallmann and his co-workers on the relationship of nervous structures to the regulation of the intraocular pressure. We did not perform any physiologic measurements, but we hope this will be done in the future.

THE EFFECTS OF SUCCINYLCHOLINE ON THE EXTRAOCULAR STRIATE MUSCLES AND ON THE INTRAOCULAR PRESSURE*

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INTRODUCTION

It is known that succinylcholine and decamethonium cause striate muscle relaxation in some species and contraction in others.[8] Zaimis demonstrated in 1951[9] that mammalian muscle which normally was not stimulated by decamethonium would go into contracture after the muscle had been denervated. Both acetylcholine and decamethonium are depolarizing agents and the effect of decamethonium is similar to that reported by Brown in 1937[1] for the action of acetylcholine on mammalian striate muscle. In 1941, Brown and Harvey[2] found that the extraocular striate muscles would first proceed into a tetanic contraction and then into a contracture with increasing doses of acetyl-

* From the Ophthalmology Branch, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare.

choline even though the muscles had not been previously denervated. It thus appeared that the extraocular striate muscles of the mammalian eye reacted to depolarizing agents in a manner similar to that of other striate muscles after denervation.

Hofmann and Lembeck[3] observed a contracture of the superior oblique muscle of the dog after the administration of either decamethonium or succinylcholine. Hofman and Holzer[4] later reported a rise of intraocular pressure in human volunteers by the use of succinylcholine. Drucher,[5] however, found that decamethonium induced no eye pressure changes in his series of unanesthetized human volunteers. The elevation of intraocular pressure induced by succinylcholine in the cat was ascribed by Lincoff et al.[7] to an increase of extraocular striate muscle tension.

The present study represents a further attempt to elucidate the mechanism by which the intraocular pressure, in cats, is increased by succinylcholine and to also determine the type of muscle response evoked. The correlation between changes of tension of the extraocular muscles and changes of intraocular pressure were studied by recording simultaneously the intraocular pressure of one intact eye and the tension of extraocular muscles from the other eye in an animal following intravenous injection of succinylcholine. Study of the mechanism by which succinylcholine increases tension of the extraocular muscles was approached by recording, concurrently, the tension itself, the electrical activity elicited by the drug and the responses produced in the muscle by stimulation of its motor nerve. Since this study was completed, Lincoff et al.[6] have reported data similar, in great part, to those reported here.

Methods employed

Young adult cats were used in these studies. The animals were either anesthetized with chloralose (20 mg./cc. 70 percent alcohol) 2.0 cc./kg. intraperitoneally, or decerebrated. In the latter case, decerebration was performed under ether anesthesia; at least one hour was allowed to elapse before measurements were made on the eye muscles. Tension and electrical activity were recorded from the inferior oblique muscle.

For this purpose, the eye on one side was eviscerated, and the tendon attachment of the inferior oblique muscle at the eye was severed. The third nerve could then be easily found and traced coursing from the muscle, close to the ciliary ganglion. The nerve was sectioned at its proximal end and used for indirect stimulation of the muscle.

In this procedure, the globe was ligated as far back in the orbit as possible and sectioned. This allowed ample space for the placement of both the stimulating electrode on the nerve as well as the pickup electrodes on the muscle, with minimal bleeding. Light mineral oil was periodically dropped over the exposed muscle and nerve to minimize drying.

Nearly isometric muscle tensions were measured by the use of a strain gauge and recorded on both a Sanborn Polyviso recorder and on a cathode ray oscilloscope. Electrical activity of the muscle was amplified by means of a Grass AC preamplifier, monitored on the oscilloscope and photographed with a Grass camera.

Injections were generally made through the left femoral vein that was previously cannulated with polyethylene tubing for this purpose. On occasions, the left common carotid artery was cannulated with a small "T" tube so that injections could be made into the artery without interfering with the blood flow to the head. Systemic blood pressure was recorded from the left femoral artery. In several experiments, venous pressure was measured from the left external jugular vein. Blood pressure and venous pressure were recorded by the use of Sanborn Electromanometers.

Eye pressure changes were measured directly by the insertion of a No.-27 hypodermic needle into the anterior chamber and

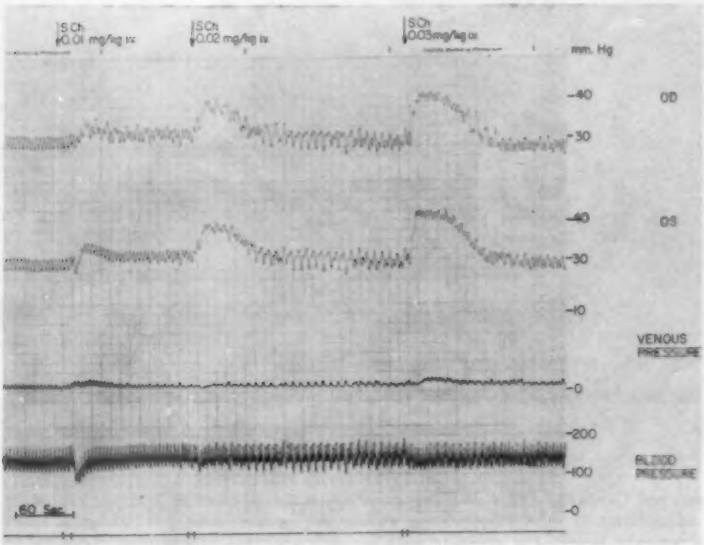

Fig. 1 (Macri and Grimes). *Effects of graded doses of succinylcholine on the intraocular pressure.* Increasing doses of succinylcholine produce proportional increases of the intraocular pressure. Frequently, but not always, there is also produced an increase of venous pressure.

recorded by means of Statham transducers. The animals were artificially ventilated through a tracheal cannula. In most experiments, the rate of indirect stimulation was at 1.0/sec.; the voltage used was adjusted to that level which produced a twitch of the muscle and a spike of submaximal amplitude.

Results

Graded doses of succinylcholine, administered intravenously, produced proportional elevations of intraocular pressure (fig. 1). Minimal responses were obtained with doses of 0.005 mg./kg. and maximal responses with doses in the range of 0.1 mg./kg. D-tubocurarine in a dose of 0.1 mg./kg. was capable of reducing the eye pressure rise brought about by 0.03 mg./kg. succinylcholine (fig. 2).

In two animals, section of the four recti and two oblique muscles of one eye was found capable of reducing, to a great extent, the intraocular pressure effect of this agent (fig. 3). A small increase of venous pressure occurred simultaneously with the residual elevation of intraocular pressure. The relationship of the venous pressure increment to the eye pressure effects was not determined.

These findings confirm those reported by Lincoff.[7]

Tension changes of the inferior oblique muscle due to succinylcholine were not modified by section of the motor nerve.

In 19 animals, electrical activity was led off from the inferior oblique muscle, indirectly stimulated through the third nerve, while simultaneously recording muscle tension changes. Succinylcholine in doses of 0.01 mg./kg. generally produced small increases of muscle tension (fig. 4) simultaneous with a small increase of intraocular pressure in the contralateral eye. Asynchronous electrical activity usually present at rest was increased in both rate and amplitude. Muscle twitch and evoked electrical discharge induced by indirect stimulation was unaffected at this dose level (fig. 5).

Succinylcholine in doses of 0.05 mg./kg., as with the lower dose, produced a burst of

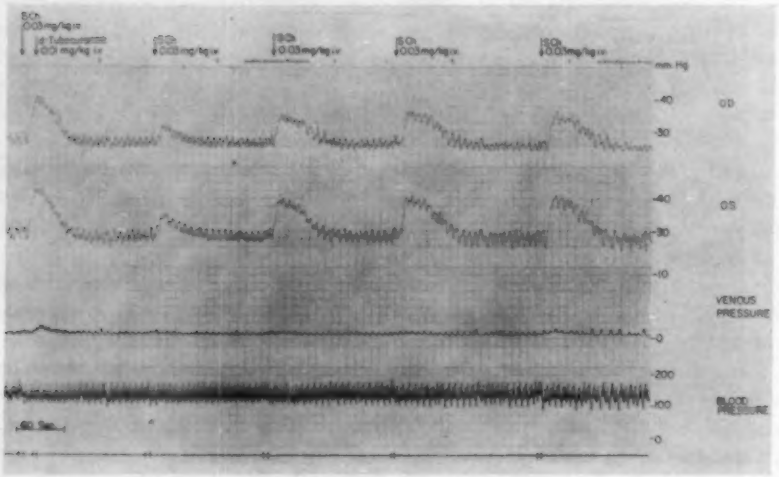

Fig. 2 (Macri and Grimes). *Effect of d-tubocurarine on the intraocular pressure responses of succinylcholine.* The administration of d-tubocurarine during the peak rise of intraocular pressure induced by succinylcholine quickly lowers the pressure back to normal levels. Subsequent doses of succinylcholine produce gradually increasing elevation of the intraocular pressure, approaching that obtained prior to the administration of d-tubocurarine. Increasing response of the intraocular pressure represents the gradual elimination of the d-tubocurarine.

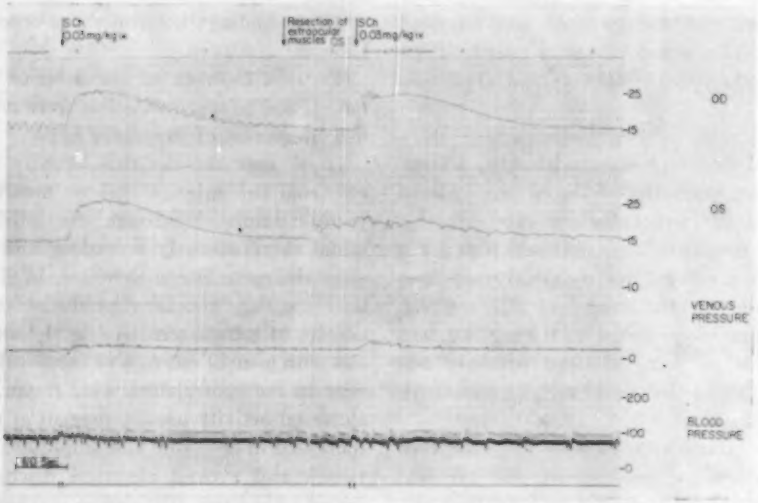

Fig. 3 (Macri and Grimes). *Effect of resection of the extraorbital muscles on the intraocular pressure response to succinylcholine.* Upper tracings indicate that the intraocular pressure elevation brought about by the administration of succinylcholine are markedly reduced after the extraocular muscles to that eye have been resected. Pressure recordings are in mm. Hg.

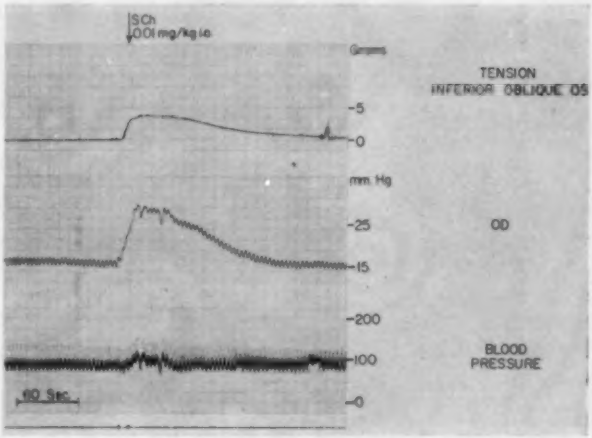

Fig. 4 (Macri and Grimes). *Effect of succinylcholine on intraocular pressure and the contralateral inferior oblique muscle tension.* Intraocular pressure increases synchronously with an increase of the muscle tension. The responses of this agent on the blood pressure vary. On occasions the blood pressure is elevated, as occurs in this record, on other occasions a depression is noted but in the majority no effects on blood pressure are found.

electrical activity synchronous with an increase of muscle tension and an elevation of intraocular pressure in the other eye (fig. 6). The spontaneous activity, though usually sustained, was reduced in magnitude soon after the peak muscle tension was reached. On most occasions the spike and muscle twitch induced by third nerve stimulation were reduced (fig. 7) and on one occasion abolished.

In doses of 0.1 mg./kg., succinylcholine markedly inhibited the muscle twitch as well as the spike elicited by nerve stimulation (fig. 8). As with the previous dose levels, this pharmacologic agent caused an initial burst of spikes concomitant with the other responses previously noted. Asynchronous activity was short-lived. Several seconds after the muscle had attained its maximum tension, activity rapidly declined to an absolute silence (fig. 9).

In three experiments, electrical activity and tension of the gastrocnemius muscle were determined simultaneously while recording the effects on the eye muscle and on intraocular pressure (fig. 10). Although the eelctrical discharge of the inferior oblique muscle occurred in the expected manner with intravenous administration of succinylcholine, no activity of the gastrocnemius muscle could be recorded (fig. 11).

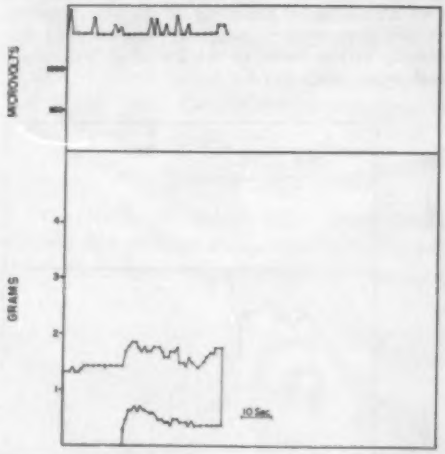

Fig. 5 (Macri and Grimes). *Effect of 0.01 mg./kg. succinylcholine, administered intravenously, on the inferior oblique muscle tension and evoked spike and muscle twitch due to stimulation of the third nerve.* Upper tracing: Ordinate: height of spike elicited by nerve stimulation. Abscissa: time. This tracing indicates that the evoked potentials due to stimulation of the peripheral trunk of the third nerve were not affected by the administration of succinylcholine at this dose level. Lower tracings: Ordinate: muscle tension. Abscissa: time. The lower line represents the steady tension of the muscle. The upper points represent the transient increases of tension occurring during muscle twitches brought about by nerve stimulation and accompanied by corresponding spikes plotted in the upper record. A small increase of steady muscle tension can be seen to occur with this dose of succinylcholine accompanied with a small alteration of the twitch tension.

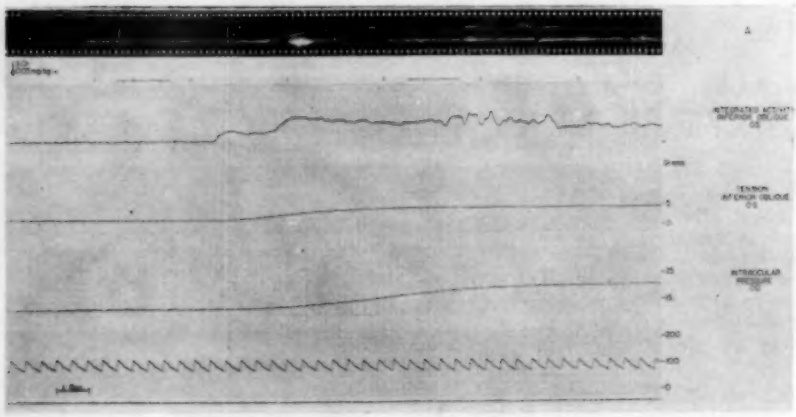

Fig. 6 (Macri and Grimes). *Synchronous response of the intraocular pressure, muscle tension, and electrical activity of the inferior oblique muscle brought about by the administration of succinylcholine.* (A) Photographic recording of the asynchronous electrical discharge of the muscle. Second line present in this photograph is muscle tension identical with that recorded on the Sanborn instrument. Integrated activity was obtained by the use of a diode and condenser and represents in another form the electrical activity as shown in (A).

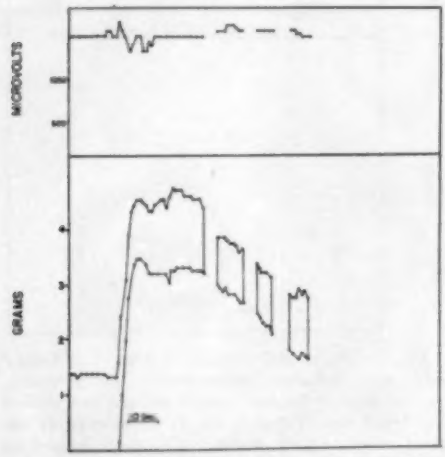

Fig. 7 (Macri and Grimes). *Effect of 0.05 mg./kg. succinylcholine, administered intravenously, on the tension and evoked spike and muscle twitch due to stimulation of the third nerve.* Plots as in Figure 5. Upper tracing indicates that the evoked potentials due to stimulation of the peripheral stump of the third nerve was not appreciably affected by the administration of succinylcholine at this dose level. Lower tracing (lower line) indicates the increase of muscle tension induced by succinylcholine. The upper line shows the twitch tension induced by nerve stimulation to be but slightly altered by this dose of succinylcholine. Stimuli were submaximal at a frequency of one/second.

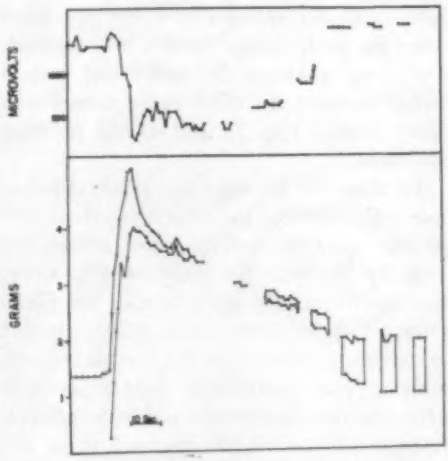

Fig. 8 (Macri and Grimes). *Effect of 0.1 mg./kg. succinylcholine, administered intravenously, on the inferior oblique muscle tension and evoked spike and muscle twitch due to stimulation of the third nerve.* Plots as in Figures 5 and 7. Upper tracing indicates that the evoked potentials due to stimulation of the third nerve were markedly depressed by the administration of succinylcholine at this level. Lower tracing (lower line) shows the marked increase of muscle tension brought about by the drug itself. Upper line demonstrates the gradual abolition and return of the twitch response. Breaks in the tracings indicate a time lapse between adjoining recordings. Time from the beginning to the end of this figure was 12 minutes. Stimuli were submaximal at a frequency of one/second.

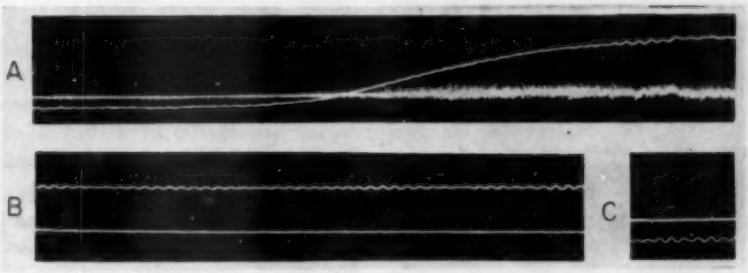

Fig. 9 (Macri and Grimes). *Effect of 0.1 mg./kg. succinylcholine, administered intravenously, on the inferior oblique muscle tension and electrical discharge.* Succinylcholine administered at the beginning of (A). Ten seconds elapsed between (A) and (B). (C) Calibration signal; 50 μ volts. Time of (A) is 4.8 seconds.

Succinylcholine administered through the common carotid artery in doses of 0.001 mg./kg., produced effects on muscle tension and electrical activity similar to those reported for 0.01 mg./kg. intravenous doses (fig. 12).

The asynchronous discharges brought about by succinycholine administration was generally recorded in the form of spikes of less than 500 μ volts, usually in the range of 100-200 μ volts. Although increasing doses of succinylcholine caused greater tensions on the extraocular muscle, very infrequently did the tension, as measured, exceed a 10-gm. pull.

The tension and electrical response of the muscle, evoked by succinylcholine, appeared similar in both the chloralose-anesthetized and decerebrate preparations.

Discussion

The results of the reported experiments confirm and perhaps extend previously available information. Succinylcholine increases

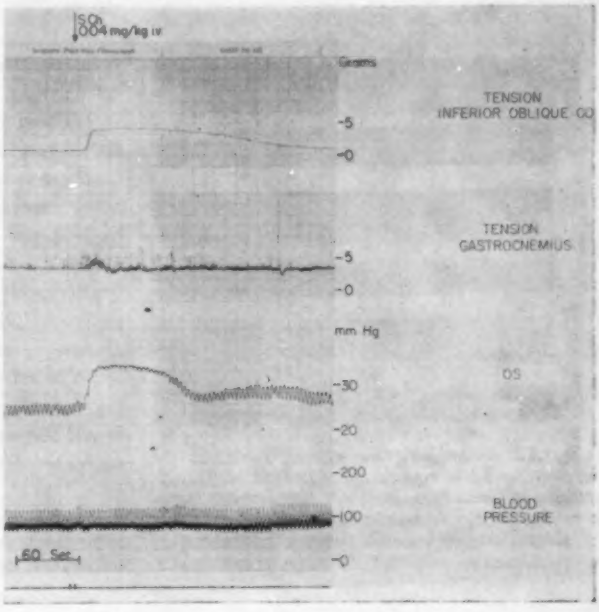

Fig. 10 (Macri and Grimes). *Comparison of succinylcholine effects on the inferior oblique and gastrocnemius muscle, intraocular pressure, and systemic blood pressure.* It can be seen that succinylcholine produced an increase of the intraocular pressure synchronous with an increase of tension of the inferior oblique muscle. No appreciable effects can be noted on the gastrocnemius muscle. The irregularity of the tracing is due to fibrillatory movements of the animal.

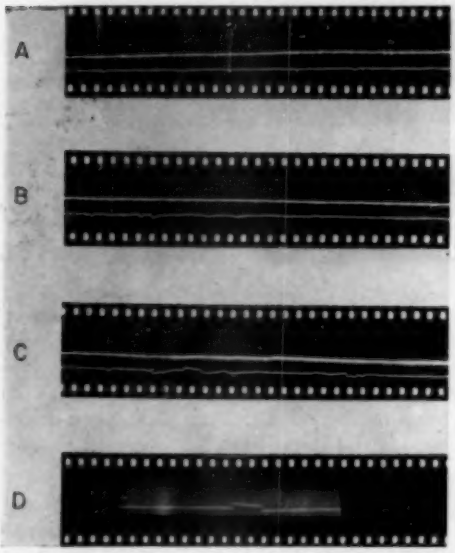

Fig. 11 (Macri and Grimes). *Comparative activity of succinylcholine on the inferior oblique and gastrocnemius muscles.* (A) (B) and (C) are consecutive recordings of the electromyograms of the inferior oblique muscle (upper tracing) and of the gastrocnemius muscle (lower tracing). Succinylcholine (0.05 mg./kg., intravenously) administered between (B) nad (C). An increase of electrical activity can be seen to occur only in the inferior oblique muscle. (D) Calibration signal—100 μ volts. Time (A) is 5.6 seconds.

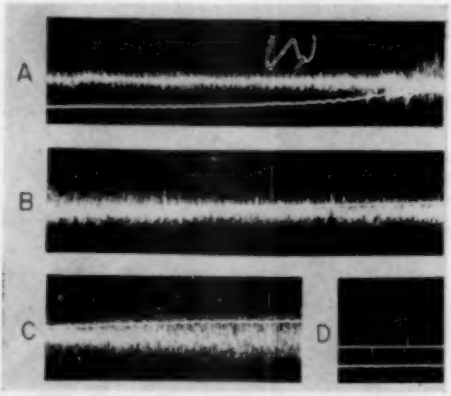

Fig. 12 (Marci and Grimes). *Responses of the inferior oblique muscle to intra-arterially administered succinylcholine.* (A, B, and C) Consecutive tracings. Administration of 0.001 mg./kg. of succinylcholine into the common carotid artery of the same side produced effects on muscle tension and electrical activity similar to those obtained with intravenous doses of 0.01 mg./kg. (D) Calibration signal; 50 μv. Time (A) is 5.6 seconds.

tension of the extraocular muscles and thereby produces an elevation of the intraocular pressure. Evidence for this conclusion is provided by (1) the parallel course of the change in tension of dissected extraocular muscles and of the intraocular pressure in the intact eye of a preparation following intravenous or intra-arterial injection of succinylcholine, (2) by the observation that increases of intraocular pressure are largely abolished when changes of muscle tension are prevented by the administration of d-tubocurarine, and (3) by the finding that when extraocular muscle effects on the eye are prevented by section of the muscles, only minimal changes of intraocular pressure are noted.

Consistent with this conclusion that the intraocular pressure following the administration of succinylcholine is due to the development of tension in the striate muscle of the eye are the findings that pressure changes are largely independent from any effect of the drug on the systemic circulation. This was shown by the simultaneous recording of intraocular and blood pressures and is confirmed by the observation that the ocular changes evoked by intravenous administration of succinylcholine can be elicited also with injection of smaller doses into the carotid artery.

Tension changes of the muscles were found not to be due to an action of the agent on the central nervous system, since the responses were essentially not modified by section of the motor nerve to the extraocular muscles.

Increase of tension of the extraocular muscles following succinylcholine administration is not necessarily accompanied by impulse activity of the muscle fibers. Following low doses, electrical activity can be led from the muscle throughout the period of increased tension, usually three to five min-

utes, indicating tetanic contraction. With higher doses, however, only an initial burst of increased electrical activity is observed and all activity subsides shortly after the muscle has reached its maximum tension. For these high doses, the major portion of the tension increase should therefore be ascribed not to a tetanic contraction but to contracture of the muscle. In this respect, succinylcholine appears to exert an action similar to that described for acetylcholine.

Summary and Conclusions

1. Succinylcholine produces an increase of intraocular pressure due to contraction of the extraocular striate muscles.

2. Low doses of succinycholine produce a tetanic contraction of the extraocular striate muscles as evidenced by increased muscle tension and electrical activity. Higher doses of this agent produce contracture of the muscles since electrical activity is lost but the tension is maintained.

Ophthalmology Branch (14).

Acknowledgment

We wish to express our thanks to Dr. M. G. F. Fuortes and Dr. Ludwig von Sallmann for their aid and helpful suggestions in this study. Thanks are also due to Mr. Joseph Brown for technical assistance.

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Discussion

Dr. W. Morton Grant (Boston): This study of Dr. Macri's on the effects of succinylcholine is most impressive to me for its technical excellence.

Earlier work on the same subject by other investigators has also been very good and seems to me to have already established satisfactorily that succinylcholine causes contraction of the extraocular muscles and thereby raises the intraocular pressure. Dr. Macri has not questioned the work of others, but, rather, he has given powerful support to its validity.

Dr. Macri has employed uncommonly elaborate and refined techniques of measurement and recording, and the results which he has presented in the figures of his article illustrate beautifully the effects of succinylcholine on the muscles and pressure of the eye. I can find nothing to criticize, but only to commend.

Among the most notable features of the action of succinylcholine is that the contractile response is peculiar to the extraocular muscles; other skeletal muscles respond in opposite manner by relaxing. It is interesting that this special response is induced by decamethonium as well as by succinylcholine, and that these two molecules resemble each other in having a pair of quarternary ammonium groups linked together by chains 10 atoms in length. Similar compounds of shorter chain length are known to cause a transient decrease of intraocular pressure, rather than a rise. It would be interesting to know by direct measurements on the extraocular muscles to what extent these muscles are influenced

by the shorter homologues.

From the clinical standpoint it is evident that the elevation of intraocular pressure induced by a single dose of succinylcholine is too brief to endanger the intact eye. However, disastrous expulsion of ocular contents is reported to have occurred when succinylcholine was administered during operation for removal of cataract.

It is interesting to speculate on the possibility of closing a narrow anterior chamber angle by contracting the extraocular muscles and thereby pushing the lens-iris diaphragm forward. It is evident that time would be required for aqueous to be lost from the anterior chamber before the lens-iris diaphragm could come forward. In this regard it would be interesting to know how long the contraction of extraocular muscles can be sustained by continuous or repeated administration of succinylcholine.

DR. F. J. MACRI (closing): I should like to comment on two points Dr. Grant brought up. One is the effect of shorter homologues of decamethonium. We have tried hexamethonium, which exhibits no activity whatever. In 1943 Brown did try acetylcholine, and he found that the activity of acetylcholine was identical to that presented here today. In low doses it does present a tetanic contraction, while with higher doses it produces a contracture.

We have not made any efforts in inducing sustained contractions with succinylcholine. All of our experiments were on an acute basis.

HISTORY OF THE ASSOCIATION FOR RESEARCH IN OPHTHALMOLOGY

CONRAD BERENS, M.D.
New York

AND

EUGENE M. BLAKE, M.D.
New Haven, Connecticut

Because a small research society devoted to ophthalmology was formed in New York in the early 1920's* and functioned successfully for a number of years, the organization of a national association was contemplated with the additional plan of seeking the cooperation of the allied sciences on a national and international basis. With this in mind, letters were written to William L. Benedict, Arthur J. Bedell, Eugene M. Blake, William C. Finnoff, Harry S. Gradle, Alan Woods, Lawrence T. Post, A. Magitot in Paris, and Sir William Lister in London. All expressed interest in the proposed society.

The senior ophthalmologists who encouraged the development of the Association for Research in Ophthalmology included: Edward Jackson, Walter B. Lancaster, Edward C. Ellett, Walter R. Parker, George E. deSchweinitz, William H. Wilmer, and William H. Wilder. These men appreciated the need for the formation of a new organization. According to some of the men contacted, however, there were already too many societies requiring membership and enough of a forum was provided by the Section on Ophthalmology of the American Medical Association, the American Academy of Ophthalmology and Otolaryngology, and the American Ophthalmological Society to make it unnecessary and undesirable to have a separate research association. Lancaster in 1947 said, when he presented a silver bowl to Conrad Berens for his work in the organization and development of the Association for Research in Ophthalmology, "I well remember the difficulties that beset . . . the conception and birth of the . . . (Association for Research in Ophthalmology). Active opposition could be met by argument but indifference is a very difficult thing to combat, and there was a lot of it. . . . (Individuals) who would overcome indifference must be armed with faith and enthusiasm. 'Enthusiasm' was Pasteur's favorite word—'Entheos,' God-given, the

* The membership included: David Webster, Henry Robertson Skeele, Martin Cohen, Mark J. Schoenberg, Edgar B. Burchell, and Conrad Berens.

divine product. And faith requires vision."

It was the belief of the younger ophthalmologists in the organizing group that ophthalmology should have a closer affiliation with scientists in fields allied to ophthalmology, such as physiology, bacteriology, psychology, physics, and illuminating engineering. Following extensive discussions an organization meeting was held in Washington, D.C., on April 30, 1928, during the American Ophthalmological Society meeting, to which 50 members of that society were invited.

At the time of this meeting, 73 ophthalmologists had signified interest in the formation of such a group. Dr. Conrad Berens acted as chairman pro tem of the meeting and William H. Wilmer was elected temporary president and Eugene M. Blake, temporary secretary. After a nominating committee was appointed, William H. Wilmer was elected president, Conrad Berens, vice-president, and Eugene M. Blake, secretary.

Two more organizational meetings were held in June, 1928, at the American Medical Association convention in Minneapolis. At this time the officers resigned because of some opposition to the slate and the younger members of the group voted to adopt an organizational pattern similar to that of the Association for Research in Nervous and Mental Diseases. There was to be a board of trustees and a commission. The board of trustees was to be composed of five members, elected for from one to five years and a new member then to be elected annually for a term of five years. The year after the chairmanship had been held by the senior trustee, he was automatically to be elected to the commission. This assured the interest of a somewhat older group of men who understood the aims of the association. The other members of the commission were appointed or re-elected annually because of their special association with the topics under discussion that year. In 1948, the constitution was revised. The board of trustees now consists of six members, elected for six years, with the secretary-treasurer serving as an ex officio member and the senior member of the board as chairman.

The first commission, appointed on April 30, 1928, included the following members:

E. V. L. Brown
W. Gordon Byers
F. Phinizy Calhoun
C. A. Clapp
George S. Derby
Edward C. Ellett
Allen Greenwood
Thomas B. Holloway
Harvey J. Howard
Lucien Howe
Edward Jackson
Arnold Knapp
Walter E. Lambert
Walter B. Lancaster
S. Hanford McKee
Walter R. Parker
George E. deSchweinitz
W. E. Shahan
F. T. Tooke
F. H. Verhoeff
John M. Wheeler
William H. Wilder
William H. Wilmer
William Zentmayer

The first chairman of the board of trustees was Alan C. Woods. The board consisted of: William L. Benedict, William C. Finnoff, Harry S. Gradle, Eugene M. Blake, and Emory Hill. Dr. Arthur J. Bedell became a trustee in October, 1929. The first secretary-treasurer was Conrad Berens, a position which he held for nine years. He was followed in office by Cecil S. O'Brien. Later Brittain F. Payne served as secretary-treasurer and, when he entered the Air Force during the second world war, the duties of the secretary-treasurer reverted to Berens.

The activities of the secretaries who carried on before and since World War II, including James H. Allen and Lorand V. Johnson, have been outstanding and have done much to add to the ever-growing prestige of the organization.

The first meeting of the Association for Research in Ophthalmology was held during the American Medical Association convention in Detroit, on June 24, 1930. The attendance totalled 135. It was decided to have

no set discussions at the meetings but that questions would be passed to the commission of older members, who would then relay them to the chairman who was usually one of the commission, for the speaker's answers.

At the first two meetings, the subjects discussed were iritis and uveitis and the papers were helpful in crystallizing some of the ideas at that time. The titles of some of the presentations were: "A review of the literature on the etiology of acute iritis." "Syphilitic iritis," "The bacteriologic and immunologic aspects of iritis," "Endogenous infections in the etiology of acute iritis," and "Evaluation of etiologic factors in acute iritis." A few years later the policy of devoting an entire program to one subject was abandoned.

In 1941, Derrick Vail stated in an editorial in THE AMERICAN JOURNAL OF OPHTHALMOLOGY that "the merit and worth of the association becomes increasingly apparent with each meeting and its position in ophthalmology is assured." The Association for Research in Ophthalmology has enjoyed a close relationship with the editors of THE AMERICAN JOURNAL OF OPHTHALMOLOGY and has appreciated the co-operation of Edward Jackson, Lawrence T. Post, and Derrick Vail. It was Edward Jackson who first suggested that the papers presented before the association be published in THE JOURNAL. Reprints were purchased from THE JOURNAL and then bound as the *Proceedings* of the association which has helped to keep the finances of the association in the black. In some instances publications were too expensive for THE JOURNAL to publish in complete form and so certain funds have been expended for this purpose when it was felt that the research warranted special recognition. Dr. William L. Benedict was the first editor and served with distinction for 15 years. In 1950, it was decided to publish all the Research Association papers as a supplement to one issue of THE JOURNAL and thus the *Proceedings* became the 13th issue of THE AMERICAN JOURNAL OF OPHTHALMOLOGY.

In 1955, another project was contemplated in co-operation with THE AMERICAN JOURNAL OF OPHTHALMOLOGY, namely, a separate section on Ophthalmic Research, devoted to the work of the association and its sections, and including abstracts of research papers.

Expansion of the association's activities was inevitable. It has not only sponsored an international organization for research in ophthalmology but has also organized five sectional groups, which meet annually in various parts of the country. The Eastern Section, which held its first organizational meeting in 1948, had an attendance of 88 individuals at its 1956 meeting in New York. The other sections include: East Central, Midwestern, Western, and Southern. The organization and development of sectional meetings of the association have added strength and prestige to the work of the association. Many of the sectional meetings have not only made real scientific contributions but have also facilitated the planning of the national programs.

Moreover, the association now has three assistant secretaries, Harvey E. Thorpe, for sections of the United States; Clement McCullough for Canada; and Moacyr Alvaro for Central and South America.

In 1953, invitations were extended to a number of eye organizations, both medical and lay, asking that representatives be sent to a joint meeting for the purpose of establishing a central committee or council for research in eye diseases and the prevention of blindness, as well as to promote public relations and to disseminate information to the public. The outcome is that there is now a National Committee for Research in Ophthalmology and Blindness, sponsored by the association. As early as 1937, the association aided certain research by grants-in-aid, for example, the study of the fiber content in

melanomas of the choroid at the Army Medical Museum in Washington, D.C., by H. C. Wilder.

The Association for Research in Ophthalmology participates in the work of the American Committee on Optics and Visual Physiology by appointing three representatives and the members of the association's board of trustees are research consultants to The Ophthalmological Foundation, Inc., which is associated for research with the New York Association for the Blind.

The association has also been able to honor and pay tribute to many of its distinguished members and others contributing to research in ophthalmology. In 1937, the association was requested by the Saint Louis Society for the Blind to nominate the recipient of the Leslie Dana Gold Medal for outstanding work in the prevention of blindness. The medal was presented several times at the annual luncheon meeting of the Association for Research in Ophthalmology.

In 1947, the Proctor Medal Fund was established by Mrs. Proctor in memory of Dr. Francis I. Proctor. This medal is awarded for continued meritorious research in the basic sciences as applied to ophthalmology. Recipients of the Proctor Medal include: Jonas S. Friedenwald, Phillips Thygeson, Ludwig von Sallmann, V. Everett Kinsey, Kenneth C. Swan, David G. Cogan, George Wald, W. Morton Grant, and Norman Ashton for this year.

Recently, the Friedenwald Memorial Committee was appointed. This group was requested by the officers of the association to establish a fund, provided by friends and admirers in honor of the late Jonas S. Friedenwald, for recognition of young men performing outstanding research in ophthalmology. John Harris received this honor for the past year.

The association has grown from 108 members in 1930 to approximately 970 in 1957. During the time that the Association for Research in Ophthalmology has been in existence, real progress has been made in controlling many of the blinding eye diseases, such as ophthalmia neonatorum, trachoma (at least in the United States of America), retrolental fibroplasia, hereditary eye diseases. Some progress is also being made in the understanding of cataract and glaucoma. The written records of the association testify that this association has played an active part in this great work, as well as in many other fields of ophthalmology.

Let us hope that we of this association will never rest peacefully until all preventable blindness is eliminated and all eye conditions and diseases which cause discomfort and disability shall have been conquered.

Those of us who had the privilege of participating in the founding of the Association for Research in Ophthalmology and in its incorporation on July 20, 1936, in the State of New York, are justly proud of it and its continuing growth. One of the principal reasons for the achievements of the association is that the governing body—the board of trustees—is not a self-perpetuating group and its members are energetic active leaders in ophthalmology and the allied sciences. If the past is prologue, the future promises magnificent accomplishments.

708 Park Avenue.

303 Whitney Avenue (11).

We wish to thank Dr. William L. Benedict and Dr. Lorand V. Johnson for their co-operation in the preparation of this history.

TWENTY-SIXTH MEETING

of the

Association for Research in Ophthalmology, Inc.

Proceedings

New York City

June 3, 4, 5, and 6, 1957

BUSINESS SESSION

The annual business meeting of the Association for Research in Ophthalmology, held in conjunction with the 26th annual meeting of the association, at the Park-Sheraton Hotel, New York, New York, on Wednesday, June 5, 1957, convened at 3:15 P.M., Dr. T. E. Sanders, chairman, presiding.

CHAIRMAN SANDERS: First, we will have the report of the secretary-treasurer.

SECRETARY LORAND V. JOHNSON: Since the complete audit of the finances of the association is printed with the Supplement, I will take time today to give you only a brief summary.

Our fiscal year ends December 31st. As of December 31, 1956, expenditures for the year were $5,364. The balance in the bank on January 1, 1957, was $7,218. The balance for the prior year was $2,590, which means a net increase in our treasury for the year 1956 of $4,627.

CHAIRMAN SANDERS: I think it appropriate at this time to call the attention of the audience to the fact that the Friedenwald fund is now in existence. There has never been any published solicitation for funds prior to the first Friedenwald lectureship, which you all heard yesterday. There is some $5,800 in this fund. Any of you who wish to contribute are welcome to do so, and the fund is now open for solicitation. Dr. David Cogan is chairman of the Friedenwald fund, and any contributions may be sent to him.

We will have the auditor's report. Dr. Newell.

DR. FRANK W. NEWELL: The auditing committee, consisting of Dr. McGavic and myself, inspected the auditor's report and believe it reflects accurately the financial condition of the association, and recommends its acceptance.

I move its acceptance.

[The motion was duly seconded, was put to a vote, and was carried unanimously.]

CHAIRMAN SANDERS: We will have the report of the trustees. Dr. Romaine.

DR. HUNTER H. ROMAINE: Mr. Chairman and gentlemen, the trustees have suggested the possibility of an interim meeting to be held in the winter, in order to take some of the stress off the present very crowded meeting of the AMA session. This is only tentative, but it is brought to your attention for consideration.

The report of the nominating committee is as follows:

For trustee, Dr. Alson E. Braley.

For secretary-treasurer, Dr. Lorand V. Johnson.

For honorary membership, Dr. Ludwig von Sallmann.

CHAIRMAN SANDER: Thank you, Dr. Romaine.

Gentlmen, you have heard the report of the trustees. Is there a motion?

VOICE: I move the report be accepted.

[The motion was duly seconded, was put to a vote, and was carried unanimously.]

CHAIRMAN SANDERS: I might say that I think the association owes its thanks to Dr. Romaine for the splendid job he has done as chairman of the local arrangements committee for this particular meeting.

If there is no further business, we are adjourned.

AUDITOR'S REPORT

Association for Research in Ophthalmology, Inc.

To the Members of The Board of Trustees
Association for Research in Ophthalmology, Inc.
Cleveland, Ohio

Dear Sirs:

I have examined the cash basis accounting records for the Association for Research in Ophthalmology, Incorporated, as of December 31, 1956, and the related transactions of receipts and disbursements for the year then ended. The examination was made in accordance with generally accepted auditing standards and included such tests of the accounting records and such other procedures as I considered necessary in the circumstances.

Despite the fact that no confirmation of dues in arrears was made directly with the members, in my opinion the accompanying statements present fairly the fund balances of the Association for Research in Ophthalmology, Incorporated as of December 31, 1956, and the receipts and disbursements for the year then ended, in conformity with generally accepted cash basis accounting principles and applied on a basis consistent with that of the prior year.

Very truly yours,

MARTIN C. BLAKE.

Cash and Securities in Funds

	General Fund	Proctor Medal Fund	Total
Cash 12/31/55	$ 2,590.19	—0—	$ 2,590.19
Cash 12/31/56	7,218.14	—0—	7,218.14
Increase	$ 4,627.95	—0—	$ 4,627.95
Securities		$10,184.63	10,184.63
Total: Cash & Securities as of 12/31/56			$17,402.77

Statement of Cash Receipts and Disbursements
For Year Ended December 31, 1956

Cash Balance on January 1, 1956		$ 2,590.14
Add—Receipts		
Prior Years' Dues:		
60 Active Members @ $5.00	$300.00	
10 Educational Members @ $2.00	20.00	
2 Educational Members @ $3.00	6.00	326.00
1956 Dues [see below]		7,877.00
1957 Dues [see below]		388.00
Banquet Proceeds		625.00
Bond Interest		250.00
Friedenwald Memorial Fund		526.25
		$12,582.39
Deduct—Disbursements		
Convention Expenses:		
Dinners	$625.00	
Programs, Notices, Tickets, etc.	578.13	
Expenses—Secretary-Treasurer	250.00	1,453.13
Publication—American Journal of Ophthalmology, 1956		1,000.00
Stationery, Supplies, Printing		353.97
Postage		30.00
Insurance—$5,000.00 Bond—Secretary-Treasurer		12.50
Bank charges		1.00
Telephone		154.75
Secretarial Assistance		1,425.00
Auditing Charges		150.00
Meeting of Board of Trustees in June, October—Breakfast		46.40
Contributions		200.00
Deposit on Banquet Hall for June meeting 1957		100.00
Proctor Medals [3]		437.50
		$ 5,364.25
Balance as of December 31, 1956		$ 7,218.14

Statement of Dues Collected

1957—Provisional			
	35 @ $10.00	$ 350.00	
	6 @ $ 3.00	18.00	$ 368.00
1957			
	2 Active Members @ $10.00	20.00	20.00
1956			
	669 Active Members @ $10.00	6,690.00	
	8 Active Members @ $5.00	40.00	
	8 Active Members @ $7.00	56.00	
	1 Active Member @ $12.00	12.00	
	1 Active Member @ $3.00	3.00	6,801.00
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Bribach, E. J.

LAWRENCE
Fleming, David G.

KENTUCKY

FT. CAMPBELL
Portfolio, Almerindo G.

LOUISVILLE
Heitger, Joseph D.
Keeney, Arthur H.
Pfingst, Harry A.

LOUISIANA

FT. POLK
Christensen, Robert E.

NEW ORLEANS
Allen, James H.
Bahn, Charles A.
Bahn, Gustav C.
Barber, Aeleta N.
Boles, William M.
Byers, Jerome L.
Clark, William B.
Ellis, George S.
Guidry, Marion A.
Haik, George M.
Leckert, Edmund L., Jr.
MacDonald, Roderick, Jr.
Manning, John W., Jr.
Noble, Bertha R.
Rosenthal, J. William
Schoel, Robert E.

MAINE

BANGOR
Osler, Jay K.

LEWISTON
Tchao, Jou S.

PORTLAND
Maier, Paul

WATERVILLE
Dennis, Richard H.
Hill, Howard F.

MARYLAND

BALTIMORE
MacLean, Angus L.
Maumenee, Alfred E.
Naquin, Howard
Patz, Arnall
Pogell, Burton M.
Rowland, Louise S.
Scholz, Roy O.
Stone, Howard H.
Woods, Alan C.

BETHESDA
Chalfant, W. Paxson, Jr.
Gunkel, Ralph D.
Hart, William M.
O'Connor, George R.
von Sallmann, Ludwig

CHEVY CHASE
King, John H., Jr.
O'Rourke, James F.

MASSACHUSETTS

BOSTON
Allen, Henry F.
Balazs, Endre A.
Beetham, William P.
Boruchoff, Saul A.
Casten, Virgil C.
Chandler, Paul A.
Chisholm, Julian F., Jr.
Cogan, David G.
Dunphy, Edwin B.
Grant, W. Morton
Gunderson, Trygve
Heath, Parker
Jakus, Marie A.
Liebman, Sumner D.
Murray, Edward S.
Regan, Charles D. J.
Regan, James J.
Schepens, Charles L.
Snyder, John C.
Varga, Laszlo
Verhoeff, F. H.
Wachendorf, Fritz A. S.

BROOKLINE
Stone, William

CAMBRIDGE
Wald, George

LOWELL
Broggi, Richard J.

NORWOOD
Ryan, William F.

SPRINGFIELD
Waugh, Richey L., Jr.

WORCESTER
Holzer, William F.
Myers, Roscoe W.
Whitney, Percy T.

MICHIGAN

ANN ARBOR
Alpern, Mathew
Falls, Harold F.
Fralick, F. Bruce
Henderson, John W.
Smillie, John W.
Wolter, J. Reimer

BAY CITY
Heuser, Harold H.

DETROIT
Burroughs, Roswell G.
Golden, Alfred
Guyton, Jack S.
Johnson, David S.
Kinsey, V. Everett
Lepard, C. W.
Lerner, Leonard H.
Pugh, Howard C.
Ruedemann, A. D.
Ruedemann, A. D., Jr.
Scarney, Herman D
Schimek, Robert A.
Sugar, H. Saul
Wachtl, Carl

JACKSON
McGarvey, William E.
Olesky, Stanley P.

SAGOLA
Dean, Alfred

TRAVERSE CITY
Weih, Jack E.

YPSILANTI
Petrohelos, Manousos A.

MINNESOTA

DULUTH
Hilding, Anderson C.

FERGUS FALLS
Palmerton, E. S.

MANKATO
Johnson, Hobart C.

MINNEAPOLIS
Fink, Walter H.
Halberg, Franz
Kantar, Bruce L.
McCannel, Malcolm A.
Schwartz, Virgil J.
Walsh, Francis M.
Wendland, John P.

ROCHESTER
Bair, Hugo L.
Benedict, William L.
Henderson, John W.
Hollenhorst, Robert W.
Martens, Theodore G.
Ogle, Kenneth N.
Rucker, C. Wilbur
Sheard, Charles
Steinmetz, Rodney D.
Winn, William E., Jr.

ST. PAUL
Burch, Edward P.
Grant, Hendrie W.
Monahan, Robert H.
O'Kane, Thomas W.
Strate, Gordon E.
Tracht, Robert H.

MISSISSIPPI

JACKSON
Johnson, Samuel B.

MISSOURI

CLAYTON
Hildreth, H. Rommel
Israel, Milton R.

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Stauffer, Harry B.

KANSAS CITY
Calkins, Larry L.
Eubank, William R.
Lemoine, Albert N.
Lemoine, Albert N., Jr.
McAlester, A. W., III
McKee, Joseph W.
Robison, James T., Jr.
Shaad, Dorothy J.

MEXICO
Rouse, David M.

ST. LOUIS
Alvis, Edmund B.
Arribas, Neva P.
Becker, Bernard
Bryan, James H.
Cibis, Paul A.
James, William M.
Luedde, Philip S.
Mattis, Robert D.
Meyer, Dexter, Jr.
Miles, Paul W.
Moses, Robert A.
Post, Lawrence T.
Post, Martin H., Jr.
Rosenbaum, Harry D.
Sanders, T. E.
Schwartz, Frederick O.
Shahan, Philip T.
Venable, Howard P.

NEBRASKA

LINCOLN
Paulson, Hubert O.
Thomas, Richard L.

OMAHA
Alliband, George T.
Filkins, John C.
Gifford, Harold
Judd, John H.
Lipp, Frank E.
Morrison, W. Howard
Rasgorshek, Robert H.
Stokes, William H.
Swab, Charles M.
Truhlsen, Stanley M.

NEVADA

RENO
Magee, George R.
Roberts, N. Craig

NEW HAMPSHIRE

BERLIN
Ingalls, Raymond G.

HANOVER
Auten, Hanford L.
Sullivan, Paul B.

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Harley, Robison D.

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Levy, Abram

CAMDEN
Meyer, George P.

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Cregar, John S.

FAR ROCKAWAY
Goldsmith, Maximilian O.

HADDONFIELD
Finley, John K.

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Nicholson, F. Peter

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Rosen, Emanuel P.
Sherman, A. Russell
Sherman, Elbert S.

OCEAN CITY
Pettit, Paul H.

PASSAIC
D'Amico, Thomas V.
Ehrenfeld, Edward
Silverstein, Arthur L.

PRINCETON
Abrams, Henry

SHORT HILLS
Fonda, Gerald E.

SUMMIT
McAlpine, Paul T.

TEANECK
Berry, A. Erwin

TRENTON
Sacks-Wilner, Erwin
Sharbaugh, George B.
Wilner, Arthur S.

NEW MEXICO

ALBUQUERQUE
Dillahunt, Jack A.
Schonberg, Albert L.

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Bedell, Arthur J.

BAY SHORE
Bussey, Frank R.

BAYSIDE
Goodstein, Seymour

BINGHAMPTON
Walling, Harry G.

BRONX
Burman, Daniel
Fleischner, Alois L.
Wincor, Henry G.

BRONXVILLE
Koch, F. L. Philip

BROOKLYN
Bonadia, Calogero
Cholst, Mortimer
Evans, John N.
Fink, Austin I.
Grassi, Anthony J.
Mandelbaum, Joseph
Rosenberg, Abner S.
Rosenthal, Benjamin C.
Sands, Abraham M.

BUFFALO
Addington, Charles H.
Bennett, Arthur L.
Brennan, James W.
Buckheit, Rudolph G.
Dellaporta, Angelos
Fial, Edward A.
Fowler, James G.
Freeman, Sheldon B.
Howard, William M.
Jones, W. Yerby
LeWin, Thurber
Luhr, Alfred F.
Luhr, John P.
Naples, Ange S.
Olmsted, K. Elizabeth P.
Reitz, Herbert R.
Schopp, Robert C.
Smallen, Benjamin

CORNING
Hoffman, Parker M.

ELMIRA
Boland, William T.

FLUSHING
Kellerman, Leo D.

GLENS FALLS
Mintz, Maxwell A.
Ryan, Edward P.

JACKSON HEIGHTS
Presto, Ernest C.
Shapley, Albert

KENMORE
Vaughan, James B.

KEW GARDENS
Goldberg, Bernard

LANCASTER
Palmer, Milton A.

LOCKPORT
Morgana, Dante J.

LONG ISLAND
Boyd, James L.
Buckley, Eugene T.

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Bassen, Edward J.
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Bloomfield, Sylvan
Bonaccolto, Girolamo
Breakey, Arnold S.
Breinin, Goodwin M.
Burns, Robert P.
Campbell, Charles J.
Carroll, Frank D.
Carter, George Z.
Castroviejo, Ramon
Chambers, A. L., II
Clark, Graham
Cole, Helen G.
Cole, John G.
Coles, Robert S.
Constantine, F. H.
Curtin, Brian J. H.
Day, Robert M.
DeVoe, Arthur G.
Dische, Zacharias
Dunnington, John H.
Epstein, Sidney S.
Esterman, Benjamin
Feinstein, Robert R.
Feldstein, Morris
Fernando, Antonio N.
Foote, Franklin M.
Friedman, Benjamin
Givner, Isadore
Gordon, Dan M.
Guy, Loren P.
Hartshorne, Isaac
Jacobson, Jerry H.
Kahan, Edmund
Kara, Gerald B.
Katzin, Herbert M.
Kaufman, Ira H.
Knapp, Arnold
Knapp, Arthur A.
Knapp, Phillip
Knighton, Willis S.
Kornzweig, A. L.
Kronenberg, Bernard
Krug, Joseph H.

Laval, Joseph
Linksz, Arthur
Locatcher-Khorazo, Deborah
Lowenstein, Otto
McLean, John M.
Macnie, John P.
Mamelok, Alfred E.
Marcus, Arthur A.
Merriam, George R., Jr.
Minsky, Henry
Norton, Edward W. D.
Paton, R. Townley
Payne, Brittain F.
Perera, Charles A.
Posner, Adolph
Ralph, Fenn T.
Regan, Ellen F.
Reese, Algernon B.
de Roetth, Andrew, Jr.
Romaine, Hunter
Samuels, Bernard
Sauer, John H.
Schachat, Walter S.
Schachne, Lewis
Schlossman, Abraham
Shafer, Donald M.
Stichevska, Olga
Smelser, George K.
Smith, Byron
Snyder, Stuart S.
Straatsma, Bradley R.
Theodore, Frederick H.
Troutman, Richard C.
Turtz, Arnold I.
Turtz, Charles A.
Tusak, Ervin A.
Webster, David H.
Weimar, Virginia L.
Wheeler, Maynard C.
Wise, George N.

NIAGARA FALLS
Goetzman, Arthur C.

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Sheldon, Maurice G.

PLATTSBURG
Siegel, Edward

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Snell, Albert C., Jr.
Sullivan, Charles T.

ROME
Reid, Frederick K.

RYE
Simonton, John T.

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Sykowski, Peter S.

SYRACUSE
Gillette, David F.
Katz, I. Herbert
McGraw, James L.
Marlow, Searle B.

UTICA
Valone, Richard J.

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Cumming, Edith L. W.

WARSAW
Leathman, John W.

WATERTOWN
Atkinson, Walter S.

WEST ISLIP
Bergmann, Robert B.

NORTH CAROLINA

ASHEVILLE
Odom, Robert E.

CHARLOTTE
Ghent, Thomas D.
Sloan, Henry L.

DURHAM
Anderson, Banks

GOLDSBORO
Bizzell, James W.

WINSTON-SALEM
Holt, Lawrence B.

NORTH DAKOTA

MINOT
Kohl, Darwin L.

OHIO

AKRON
Krichbaum, Franklin M.

ALLIANCE
King, George L.

ASHTABULA
Streicher, Carl J.

CINCINNATI
Abrahamson, Ira A.
Abrahamson, Ira A., Jr.
Ascher, Charles K. W.
Brown, Albert L.
Lyle, Donald J.
McGowan, William L.
Nelson, Robert C.
Reid, Horace W.
Sakler, Barnet R.
Schrimpf, Cyril E.

CLEVELAND
Ballintine, Elmer J.
Bell, Richard P., Jr.
Bennett, James E.
Brandwan, Samuel R.
Budd, Francis X.
Chamberlain, W. P., Jr.
Gans, Jerome A.
Gans, Morris E.
Guth, Sylvester K.
Hardesty, Hiram H.
Hare, James
Harvey, Elmer F.
Hertner, John E.
Johnson, Lorand V.
Kennedy, Roscoe J.
Keyes, John E. L.
Kirk, Robert C.
Kottler, Saul
Motto, Paul
Nicholl, Russell J.
Nosik, William A.
Phillips, Josephine D.
Potts, Albert M.
Rosner, Robert S.
Schwarz, Gerald T.
Thomas, Charles I.
Volk, David
Waitzman, Morton B.
Wolpaw, Benjamin J.

COLUMBUS
Battles, Morris L.
Brecher, Gerhard A.
Ellerbrock, Vincent J.
Fry, Glenn A.
Havener, William H.
Knox, George W.
Lipetz, Leo E.
Magnuson, Robert H.
Makley, Torrence A.
Prince, Jack H.
Stine, George T.
Suie, Ted
Taylor, Frank W.
Westheimer, Gerald

DAYTON
Insel, Herman H.
Teed, Roy W.
Thomas, Edward R.

LAKEWOOD
Ellenberger, Carl

NEWBURY
Frohring, William O.

PARMA
Graham, John H.

SEVEN HILLS
Gilger, Anita P.

TOLEDO
Schmerl, Ernest
Skow, John D.
Steinberg, Bernhard
Vesey, Frank A.
Winger, Ira B.

UNIVERSITY HEIGHTS
Weinberg, Herman C.

WARREN
Chickering, Donald H.
Thomas, John H.

YOUNGSTOWN
Bloomberg, Louis
Evans, William H.
Ruth, Paul E.
Sovik, William E.

ZANESVILLE
Letson, Holton C.

OKLAHOMA

NORMAN
Wiley, George A.

TULSA
Lee, Otis S.
Ozment, Thomas L.

OREGON

COOS BAY
Flanagan, Roger M.

EUGENE
McCallum, George C.

PORTLAND
Allen, Aurelia J.
Browning, Charles W.
Butler, J. B. V.
Christensen, Leonard
Gruber, Louise
Harris, John E.
Heinrich, Daniel
Markley, Richard E.
Reeh, Merrill J.

Swan, Kenneth C.
Taylor, E. Merle

ROSEBURG
Unruh, John W.

PENNSYLVANIA

ALLENTOWN
Beitel, Robert J., Jr.
Owens, William C.

ARDMORE
Vogel, Adolph W.

BEDMINSTER
Duane, Thomas D.

BELLEFONTE
Covey, John K.

BERWICK
Ross, Joseph V. M.

BRYN MAWR
McGavic, John S.

ERIE
Alberstadt, Norbert F.

GETTYSBURG
Wickerham, Earl P., Jr.

GLENSHAW
Shaw, Harry E.

HARRISBURG
Martz, George E.

LANSDALE
Carmichael, Paul L.

MORRISTOWN
Tait, Edwin F.

PHILADELPHIA
Adler, Francis H.
Ashodian, Mila J.
Askovitz, Samuel I.
Blazar, Howard A.
Brav, Solomon S.
Cowan, Alfred
DeLong, Samuel L.
Ellis, Richard A.
Gettes, Bernard C.
Gowens, Harry L., Jr.
Green, Harry
Hallett, Joseph W.
Krewson, William E., III
Leopold, Irving H.
Lutman, Frank C.
Maynard, Robert E.
McDonald, Philip R.
Mullen, Carroll R.
Reese, Warren S.
Scheie, Harold G.
Spaeth, Edmund B.
Tassman, Isaac S.
Trueman, Robert H.
Waldman, Joseph
Zentmayer, William

PITTSBURGH
Berk, Myles M.
Billings, Robert J.
Black, Henry H.
Bronk, Henry N.
Davies, Robert H.
Dunbar, John C.
Evans, Thomas M.
Everett, William G.
Goldberg, Sol
Grana, Phillip C.
Henry, Edgar S. H., Jr.
Hoffman, Franklin D.
Krieger, A. A.
Linn, Jay G., Jr.
Linn, Jay G., Sr.
McCaslin, Murray F.
Novak, Joseph F.
Rohm, Robert F.
Steinberg, Abraam
Thorpe, Harvey E.
Weisser, Charles W.
Wolken, Jerome J.

READING
Craig, Paul C.
Koven, Arthur L.

SHARON
Bloomenthal, Sanford R.
Riddle, Ransford J.
Snyder, M. Wilson

SUNBURY
Ball, Donald N.

WARREN
Ball, William L.

RHODE ISLAND

PROVIDENCE
Stephens, H. Frederick

SOUTH CAROLINA

BEAUFORT
Sullivan, John J.

COLUMBIA
Bristow, Walter J.

SOUTH DAKOTA

HURON
Kilpatrick, William R. J.

YANKTON
Willcockson, Thomas H.

TENNESSEE

KNOXVILLE
Benedict, Walter H.

MEMPHIS
Krause, Arlington C.
Lewis, Phillip M.
Rychener, Ralph O.

NASHVILLE
Bounds, George W., Jr.
Zerfoss, Kate S.

TEXAS

AMARILLO
McKay, Edward D.

AUSTIN
Hilgartner, H. L.
Key, Samuel N., Jr.
Legett, Carey, Jr.

CORPUS CHRISTI
Gill, E. King

DALLAS
Browning, Carroll W.
Cary, Edward H.
Grafton, Edwin G.
Meisenbach, Albert E., Jr.
Melton, Carlton E., Jr.
Quinn, Lester H.
Thomas, Maxwell

EL PASO
Caylor, Robert N.
Schuster, Stephen A.

FT. HOOD
Chambers, Arthur L., II

FT. WORTH
Conner, William C.

GALVESTON
Gingrich, Wendell D.
Potter, William B.

HOUSTON
Daily, Louis, Jr.
Goar, Everett L.
Griffey, Edward W.
Leigh, Richard, Jr.
Vanzant, Thomas J.

JACKSONVILLE
Stolzar, Irwin H.

KERRVILLE
Stevenson, Roger

MCALLEN
Frink, Berton F.

PORT ARTHUR
Heare, Louis C.

RANDOLPH FIELD
Fixott, Richard S.

SAN ANTONIO
Matthews, John L.
Mims, James L., Jr.

TEMPLE
Veirs, Everett R.

WICHITA FALLS
Mansur, Harl D., Jr.

UTAH

LOGAN
Lightfoot, Vernon F.

PROVO
Oaks, Lewis W.

SALT LAKE CITY
Smith, Homer E.

VERMONT

BURLINGTON
Cunningham, John C.

VIRGINIA

RICHMOND
Guerry, duPont, II
Sheppard, L. Benjamin

ROANOKE
Stone, W. Conrad
Young, Charles A.
Young, Charles A., Sr.

WINCHESTER
McGuire, William P.

WASHINGTON

BREMERTON
Benson, Clifton E.

LONGVIEW
Hill, Robert V.

SEATTLE
Jensen, Carl D. F.
Lyda, Wood
Peter, Philip A.

SPOKANE
de Roetth, Andrew F. M.

TACOMA
Golden, Stanley D.
Ward, James F.

WALLA WALLA
Hogenson, Clifford D.

Stevens, Ralph W.

WENATCHEE
Miller, Claude K.

WEST VIRGINIA

HUNTINGTON
Esposito, Albert C. M.
Polan, Charles M.

LOGAN
Scott, Robert K.

MORGANTOWN
Ryan, Ralph W.

WISCONSIN

LA CROSSE
Mansheim, Bernard J.

MADISON
Buesseler, John A.

MILWAUKEE
Blankstein, Samuel S.
Haessler, F. Herbert

RACINE
Kadin, Maurice

FOREIGN COUNTRIES

ARGENTINA

CORDOBA
Urreta-Zavalia, Alberto, Jr.

HAWAII

HONOLULU
Holmes, William J.
Pang, Herbert G.

PUERTO RICO

PUERTE DE TIERA
Carrasquillo, Honorio F.

SANTURCE
Buxeda, Roberto
Margarida, Carlos J.

CANADA

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Duggan, John W.
Marshall, M. R.

WINNIPEG
Elvin, Norman L.

KINGSTON
Rosen, David A.

LONDON
Dyson, Charles

NIAGARA FALLS
Eager, R. F.

TORONTO
Elliot, Alfred J.
Levitt, Herbert
McCulloch, Clement
MacDonald, Roy K.
Ormsby, Hugh L.
Shusterman, Morris

MONTREAL
Boxer, Louis
Brault, Jules
Cloutier, Roland
Lerman, Sidney
Locke, John C.
Viger, R. J.

VANCOUVER
Mallek, Howard
Patterson, John W.

VICTORIA
Wiggins, Robert L.

BRAZIL

BAHIA
de Andrade, Cesario

MINAS GERAIS
Dasilva, Antonio I.

SÃO PAULO
Alvaro, Moacyr E.

CANAL ZONE

ANCON
Mahaffy, Steve R.

CUBA

HAVANA
Ferrer, Horatio

NICARAGUA

MANAGUA
Cuadra, Marie K.

PERU

LIMA
Barrere, Luciano E.
Raffo, Julio C.

VENEZUELA

CARACAS
Grom, Edward

PHILLIPINE ISLANDS

MANILA
De Ocampo, Geminiano

QUEZON CITY
Quilala, Francisco V.

BELGIUM

GHENT
François, Jules

ENGLAND

LONDON
Duke-Elder, Lady Phyllis
Duke-Elder, Sir Stewart

FINLAND

HELSINKI
Koistinen, Aune M.

ITALY

CAGLIARI
Grignolo, Antonio

SOUTH AFRICA

JOHANNESBURG
Taylor, Israel B.

SWITZERLAND

GENEVA
Blum, John

TURKEY

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Sezer, F. Necolet